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The Metabolism of the Oral Flora

2. THE OXIDATION OF SOME SUGARS BY MIXED HUMAN SALIVA

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(*Received* 28 *April* 1953)

The breakdown of carbohydrate by the oral micro-organisms has been the subject of numerous investigations. It is believed by many that the intra-oral production of acids from carbohydrate, arising from incomplete oxidation, is a factor in the production of dental caries. With a view to finding the agent causing acid production, specific organisms have been isolated from the mouth and their biochemical behaviour has been studied; this work has been outlined in a previous paper (Hartles & McDonald, 1950). More recently, the anaerobe *Clostridium welchii*, which can break down glucose into lactic acid more rapidly than does *Lactobacillus acidophilus* (Hartles & McLean, 1952), has been found in carious teeth (Hartles & McDonald, 1951; Evans & Prophet, 1951). Undue emphasis must not be placed on the results obtained by studying cultures of organisms isolated from the mouth, for the presence of a particular acidogenic organism may be relatively unimportant if enzymes which can complete the oxidation of the carbohydrate are contributed to the system by other organisms. For example, lactic acid may be the end point of glucose metabolism for an *in vitro* culture of *Lb. acidophilus*, but it certainly is not so in the mouth, for frequently other organisms exist there which can metabolize lactic acid (Douglas, 1950; Neuwirth & Summerson, 1951). Fosdick & Hansen (1936) were perhaps the first to realize fully the importance of this symbiotic relationship. Thus, whilst recognizing the contribution which has been made by the investigation of specific bacteria, we consider it desirable to study the enzymic properties of saliva as a whole. We have already studied the oxygen uptake of saliva samples with and without the addition of glucose (Hartles & McDonald, 1950); a similar study by Neuwirth & Summerson (1951) reached substantially the same conclusions. It seemed desirable to extend this work using other sugars, primarily to find out if there were any differences in the rates of oxygen uptake by a series of salivas when each sugar was added separately. The sugars chosen were glucose, fructose, maltose, and sucrose, since they are the most relevant to man. We found that in the saliva of all the subjects studied, fructose was metabolized less rapidly than the other three sugars.

EXPERIMENTAL

Material. Samples of saliva were obtained from ten volunteers with varying caries experience.

Collection of saliva. The method of collection of early-morning saliva was that described by Hartles & McDonald (1950).

Manometry. The saliva samples (0·5 ml. or 1·0 ml., depending on the enzymic activity) were buffered with 0·2M phosphate buffer, pH 7·4. O_2 consumption was measured in Warburg manometers at 38°. The final concn. of sugar, when used, was 0·005M. Increasing the concn. to 0·01M had no noticeable effect on respiration. The difference in O_2 uptake between saliva incubated alone and with added sugar was a measure of oxidation of the sugar.

RESULTS

The oxidation rates of the four sugars are shown in Table 1. The subjects have been arranged in two groups of five in order of increasing DMF number (i.e. the number of teeth decayed, missing or filled); those in group I have an average DMF of 8 and those in group II of 19.

In group I fructose and maltose showed a consistently significant difference in metabolic rate for each subject; the difference between fructose and sucrose was significant for two subjects, J.M. and J.R.D. ($P < 0·05$), while that between glucose and fructose was not significant at the 5 % level. The analysis of variance shows that combining the data for all five subjects accentuates the difference between sugars; fructose is metabolized at a significantly lower rate than any of the other three sugars ($P < 0·001$), whilst maltose is metabolized at a significantly higher rate than either glucose ($P < 0·001$) or sucrose ($P < 0·01$). There is no significant difference in the rates of oxidation of glucose and sucrose.

Results for group II show that each subject (except M.R.W. with sucrose) metabolizes glucose, maltose, and sucrose, at a significantly higher rate than fructose; also, that the variation in the values for each sugar is greater than that displayed by the subjects of group I. This is established by an analysis of variance of the combined data for group II in which the residual variance used to assess the

Table 1. *Mean oxygen consumption of salivary samples due to the addition of different sugars*

(All values expressed as μl. O_2/ml. saliva/hr. Final concn. of added sugar, 0·005 M. Temp. 38°.)

Subject	Teeth decayed, missing, or filled (DMF)	Glucose	Fructose	Maltose	Sucrose	*S.E.	No. of days
			Group I				
S.F.S.	4	91·7	87·1	98·5	91·1	4·0	12
J.M.	6	68·5	62·9	77·5	77·8	4·0	10
J.G.	8	63·7	59·6	74·7	63·0	3·1	12
J.R.D.	10	180·4	170·2	186·7	186·3	5·8	12
A.G.L.	12	143·0	132·4	149·8	140·5	5·7	11
Mean	8	109·4	102·4	117·4	111·7	—	—
Weighted mean		110·2	103·4	118·1	112·5	—	—
			Group II				
R.L.H.	17	397·1	334·8	390·2	411·5	14·7	13
M.R.W.	17	136·4	113·5	128·9	121·4	5·1	14
L.G.	18	108·0	77·5	103·1	110·8	5·1	10
I.A.R.	20	60·6	46·8	68·0	65·0	3·9	11
P.D.B.	23	134·6	115·5	138·5	135·6	5·2	12
Mean	19	167·3	137·6	165·7	168·9	—	—
Weighted mean		173·9	143·3	172·0	175·0	—	—

* S.E. is the standard error of the difference between any two means: thus, roughly speaking, a difference of more than twice this value between any two means in a given row is statistically significant.

significance of the variance between sugars is much greater than that in the analysis for group I. Thence it can be seen from the overall analysis for group II that, again, fructose is the sugar least rapidly metabolized ($P < 0·001$), but that there is no significant difference in the rates of metabolism of the other three sugars.

An analysis of variance of the whole data for the ten subjects shows that the difference in metabolic rate between fructose and the other sugars is highly significant ($P < 0·001$). When deliberate selection is exercised and the subjects are arranged in two groups according to their DMF numbers, certain tendencies are observed. The differences between sugars with subjects of lower DMF (group I) are sharpened, because the general pattern of metabolism amongst these subjects is the same, that is, that fructose is metabolized less and maltose more rapidly than the others, whilst there is no significant difference between rates of glucose and sucrose metabolism. On the other hand, small differences between sugars with subjects of higher DMF (group II) are blurred because these subjects tend to behave differently toward different sugars and thus the only significant difference in this group is that between fructose and the three other sugars.

DISCUSSION

Dixon & Needham (1946) reported that yeast hexokinase had a lower affinity for fructose than for glucose. Meyerhof & Wilson (1949) obtained similar results for brain hexokinase. Slein, Cori & Cori (1950) found that yeast and brain hexokinases were similar in many respects, and they produced evidence to show that a single enzyme was responsible for the action on both glucose and fructose. Our results suggest that a hexokinase is concerned in the oxidation of glucose and fructose by the oral flora.

We found that sucrose was, in all except one of the subjects, metabolized at least as rapidly as glucose by the saliva. There is some disagreement in the literature concerning the existence of invertase in saliva (Bourquelot, 1910; Lisbonne, 1910; Tauber & Kleiner, 1933; Wohrinz, 1938; Volker, 1950). The fact remains, however, that unfiltered whole saliva can metabolize the disaccharides sucrose and maltose no less readily than glucose. Doudoroff, Kaplan & Hassid (1943) demonstrated that *Pseudomonas saccharophila* contained an enzyme which phosphorylated sucrose directly. There are, then, two possible pathways for sucrose breakdown. If sucrose is acted upon by invertase to produce glucose and fructose in equimolecular proportions, and if glucose and fructose are competing for hexokinase, it is not unreasonable to expect sucrose to be oxidized at a rate significantly less than glucose. For our group of salivas this was not so, and our results suggest that preliminary hydrolysis of sucrose may not be the first step in its metabolism. Further work is necessary to decide this point, and also whether the non-glycolytic enzymes concerned are a secretion of the salivary glands or are contributed by the oral flora. The only salivary carbohydrase which has been shown to be a product of the salivary glands is α-amylase.

Roberts & Whelan (1951) have shown that this enzyme, when purified, has no action on maltose.

We are not at this stage particularly concerned with differences between 'caries active' and 'caries inactive' groups of subjects. We also realize that the present DMF number is not necessarily an indication of the present caries activity. Nevertheless, certain interesting observations can be made. In group I (low DMF) fructose is metabolized at a significantly lower rate than any of the other sugars. There are also significant differences in the rates of oxidation of maltose and sucrose, and of maltose and glucose. There is no such difference in the case of glucose and sucrose. In group II (high DMF) the only conclusion which can be drawn is that fructose is metabolized at a lesser rate than the other three sugars. Our results therefore suggest that the pattern of utilization of different sugars by group I subjects differs from that of group II subjects. It is, however, unwise to deduce more from these observations on so few subjects.

SUMMARY

1. The salivary flora metabolize fructose at a significantly lower rate than glucose, maltose, or sucrose.

2. Subjects with a comparatively low DMF number (number of teeth decayed, missing or filled) also show differences in oxidation rate between glucose and maltose, and between sucrose and maltose, but not between sucrose and glucose.

3. Subjects with a high DMF number did not show the differences enumerated in paragraph 2.

One of us (M.R.W.) wishes to acknowledge the receipt of a full-time grant from the Medical Research Council. The authors wish to express their thanks to Mr K. C. T. Gordon, Department of Applied Mathematics, University of Liverpool, for his valuable advice and for his help in some of the statistical analyses. They are also indebted to the volunteers who provided saliva samples cheerfully over an extended period.

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The Breakdown of Urea in Cats not Secreting Gastric Juice

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Although Luck (1924*a*, *b*) had shown urease to be present in the stomachs of a wide variety of animals, urea was until recently regarded as solely an end-product of mammalian nitrogen metabolism. Experiments with [^{14}C]urea (Leifer, Roth & Hempelmann, 1948; Langham, 1949; Jones, 1950; Hastings, Langham, Carter & Roth, 1950; Skipper *et al.* 1951; Kornberg, Davies & Wood, 1953*a*) and [^{15}N]urea (Davies & Kornberg, 1950; Von Korff, Ferguson & Glick, 1951; Kornberg & Davies, 1952*a*, *b*) showed, however, that cats, dogs, mice, and guinea pigs, could hydrolyse urea *in vivo* and that urea was therefore not metabolically inert.

The experiments reported in this and a subsequent paper (Kornberg, Davies & Wood, 1954) were undertaken to study the rate of, and the conditions affecting, the breakdown of [^{14}C]urea in cats, whose stomachs were allowed either to rest or to secrete gastric juice. Previous investigations of the behaviour in the cat of [^{14}C]carbon dioxide liberated into the blood (Kornberg, Davies & Wood, 1952*a*) had shown that the amount of [^{14}C]urea hydrolysed

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in vivo could be determined from the amounts of [^{14}C]carbon dioxide produced and subsequently expired. This procedure was used in the present work. In the earlier studies of urea breakdown in mice (Leifer *et al.* 1948; Jones, 1950; Hastings *et al.* 1950; Skipper *et al.* 1951) and guinea pigs (Langham, 1949), the experimental animals were placed in respiration chambers after injection of [^{14}C]urea, and the quantity of urea hydrolysed was also determined from the quantity of [^{14}C]carbon dioxide expired. This procedure did not allow of any surgical interference with the animals during experiments or the taking of blood and tissue samples, and the present work was therefore carried out with cats attached to a special respiration circuit (Kornberg *et al.* 1952*a*) by a tracheal cannula, leaving the rest of the animal accessible. By this technique, information was obtained on the rate of urea breakdown in the cat, and on the site of this breakdown in the animal.

Part of this work has been communicated to the Biochemical Society (Kornberg, Davies & Wood, 1953*b*).

EXPERIMENTAL AND RESULTS

General. The general experimental techniques used in the treatment of cats, in the collection and analysis of respiratory, blood, and tissue samples, and in the measurement of radioactivity, were those described previously (Kornberg *et al.* 1952*a*, 1953*a*). The special treatments employed in some experiments are described later. All cats were fasted overnight.

Labelled urea. The [^{14}C]urea used, synthesized according to Murray & Ronzio (1949), was obtained in two consignments each of 1 mc. from the National Chemical Laboratory, Teddington, and the Radiochemical Centre, Amersham, respectively.

The first consignment was received as the crude, final reaction mixture. The urea was isolated, after addition of A.R. urea as carrier, by crystallization from propanol, and was purified by recrystallization from *iso*pentanol. The m.p. was 132° (uncorr.), and the mixed m.p. 132° (uncorr.). The urea (415 mg.) was dissolved in 15 ml. of water; 1·5 ml. of this solution (containing 41·5 mg. of urea and giving 2130000 counts/min. under the conditions of counting employed) were used for any one cat experiment.

The second consignment was received as 27·4 mg. of pure urea. It was dissolved in 15 ml. of water; 1 ml. of this solution (containing 1·83 mg. of urea and giving 2030000 counts/min.) was used for any one cat experiment. The acid-volatile $^{14}CO_2$ of the [^{14}C]urea solutions, which were stored at −12°, was determined before each experiment and was always less than 0·005% of the total radioactivity.

Determination of gastric urease activity. The urease activities of cat gastric mucosae were measured manometrically. The stomach was removed from the cat, opened, and washed rapidly under running, cold water. It was then transferred to a bacteriological inoculating cabinet. The subsequent treatment of the tissue was carried out in this container, using only sterile solutions and sterile instruments, to prevent contamination of the tissue with bacteria. The mucosa was scraped off with a microscope slide, weighed, and ground with one-tenth of its weight of acid-washed sand and twice its weight of water. Of the resultant suspensions, 1 or 2 ml. were pipetted into the main compartment of a double-armed Warburg vessel, together with 2 or 1 ml. of water and 0·2 ml. of 3M-sodium acetate buffer, pH 5·0. Into the first side arm were placed 0·4 ml. of 0·8M urea and 0·1 ml. of 3M sodium acetate buffer, pH 5·0; into the second side arm was pipetted 0·5 ml. of jack bean urease solution, prepared as described previously (Davies & Kornberg, 1951).

The vessel was attached to a Warburg microrespirometer, the system equilibrated at 38°, and the contents of the first side arm mixed with those of the main compartment. The evolution of gas due to the hydrolysis of urea was measured for 30–45 min., after which time the contents of the second side arm were mixed with those of the main compartment. The immediate evolution of gas at this stage showed that any absence of gas output after the first mixing was due to the absence of urease activity in the mucosa, and not to experimental errors.

Units. The urease activity of the mucosa was expressed as Q_{urea} (μl. CO_2 evolved/mg. dry wt./hr., where 22·26 μl. of CO_2 at s.t.p. are equivalent to 1 μmole of urea).

Assay of bacterial content. Through the kindness of Dr J. K. A. Beverley, of the Department of Bacteriology, University of Sheffield, it was possible to make a bacteriological examination of the samples of gastric mucosa, obtained as described above, and of samples of various tissues removed from the cats throughout the experiments. Small portions of these samples were inoculated into aerobic and anaerobic media, the constitutions of which were as follows. *Aerobic KU medium*: Pronutrin casein hydrolysate (Herts Pharmaceuticals Ltd., Welwyn Garden City), Lab-Lemco beef extract (Oxo Ltd., London), Bacto yeast extract (Difco Laboratories Inc., Detroit), and urea. Each constituent had a final concentration of 1% (w/v). Samples (20 ml.) of this solution were adjusted to pH 7 with 0·05N-NaOH, placed in 500 ml. conical flasks, inoculated and kept at 37° for 48 hr. *Anaerobic KU medium*: a narrow, screw-cap bottle was completely filled with 10 ml. of the aerobic KU medium, described above, and 5 g. cooked, minced ox heart. The inoculated mixture was similarly kept at 37° for 48 hr.

Samples from those liquid cultures in which growth had occurred were inoculated on to plates of the aerobic KU medium solidified with agar and containing in addition 0·02% of phenol red. These plates were incubated at 37° for 48 hr., aerobically in the case of the aerobic cultures and both aerobically and anaerobically in the case of the anaerobic cultures. The presence of an alkaline zone around a colony indicated that the colony contained urease. Since none of the obligate anaerobes contained urease, the urease-positive colonies were inoculated into 20 ml. of a liquid medium contained in a 250 ml. conical flask and similar in composition to the aerobic KU medium save that it contained no urea. The flasks were incubated aerobically for 48 hr. at 37°.

The urease activities of samples of these cultures and of the bacteria-free supernatants obtained after centrifugation were subsequently measured manometrically. All the cultures grown from the urease-positive colonies contained urease, although urea had been omitted from the final medium in order to reduce the blank in the urease estimation.

The 'urea space' of the cat

When [^{14}C]urea was injected intravenously into cats, the specific activity of the blood urea rose sharply. As the labelled urea mixed with the urea in the rest of the body, the specific activity fell rapidly until, some 30 min. after the injection, the rate of fall lessened. At about 60 min. after the injection, the mixing of labelled and unlabelled urea appeared to be complete and the specific activity of the blood urea after this time decreased only slowly (by about 5%/hr. of the value at 60 min.). This fall in the later stages of the experiments was due to the dilution of the labelled urea with unlabelled urea synthesized in the liver; since the ureters were tied in all experiments, this dilution manifested itself by a steady rise in the concentration of urea in the blood. The results of two typical experiments are given in Table 1. After injection of the labelled urea, the counts/100 ml. of blood fell rapidly until, at approximately 60 min., constant values were reached and maintained for the remainder of the experiments. The radioactivities at these levels were 194000 and 108000 counts/min./100 ml. of blood for the two cats, i.e. 211000 and 117000 counts/min./100 ml. of plasma water, respectively. Since in each case 2130000 counts/min. had been injected, the labelled urea had thus been diluted with unlabelled urea dissolved in 1010 ml. (cat 1) and 1820 ml. (cat 2) of body water; the urea space of cat 1 (1·57 kg. body weight) therefore was 64·5% of the body weight, and that of cat 2 (2·80 kg. body weight) was 65·0%. These values, previously reported by Kornberg, Davies & Wood (1952*b*), are very similar to those found simultaneously and independently by Eggleton (1951).

The expiration of [^{14}C]carbon dioxide

After intravenous injection of [^{14}C]urea into cats, [^{14}C]carbon dioxide appeared rapidly in the expired air. For the first 30–40 min. after administration of the isotope, the specific activity of the expired carbon dioxide rose sharply, reaching a constant or slightly rising level after this time (Fig. 1). The total amount of [^{14}C]carbon dioxide expired, which varied with the size of the experimental animal,

Table 1. *Changes in the concentration and specific activity of blood urea after intravenous injection of* [^{14}C] *urea*

(Cat 1; lean male cat, 1·57 kg. body wt.; cat 2; male cat, 2·80 kg. body wt. Ureters tied 5 min. before injection of 2130000 counts/min. at 0 hr.)

Cat no.	Time after injection (hr. min.)	Concentration of urea (mg./100 ml. whole blood)	Specific activity of blood urea (counts/min./mg. C)	Radioactivity of whole blood (counts/min./100 ml.)
1	0 0	29·6	0	0
	0 4	39·7	55 700	442 000
	0 31	37·5	28 800	216 000
	0 59	38·8	24 800	192 000
	1 26	38·8	25 500	198 000
	1 49	40·7	24 000	196 000
	2 16	42·2	22 200	187 000
	2 50	46·7	21 300	199 000
	3 21	44·2*	20 400	180 000
	3 48	47·7	19 400	185 000
	4 30	47·7	19 200	183 000
	4 51	51·8	18 000	185 000
2	0 0	35·9	0	0
	0 5	40·7	28 900	235 000
	0 20	40·5	14 100	114 000
	0 35	42·9	12 400	107 000
	0 50	47·4	10 500	99 600
	1 05	49·1	10 700	105 000
	1 20	55·4	9 210	102 000
	1 35	55·2	8 720	96 300
	1 50	57·5	8 800	101 000
	2 10	57·5	9 100	105 000
	2 21	57·7	8 440	97 500
	2 35	59·9	8 440	101 000
	2 50	65·3	8 320	109 000
	3 05	68·8†	7 960	109 000
	3 20	68·8	7 740	107 000
	3 35	68·1	8 200	112 000
	3 50	69·4	7 830	109 000
	4 05	70·3	6 280	88 400

* 20 ml. saline injected at 3 hr. 20 min. † 15 ml. saline injected at 3 hr.

Table 2. *Urea breakdown in cats not secreting gastric juice*

(In these experiments, carried out on cats whose ureters were tied before injection of [^{14}C]urea at 0 hr., the concentration of urea in the body fluids rose throughout, and the average urea content was taken to be 7 m-moles/l. of body water. The total body water was 65% of the body weight.)

Cat no.	Wt. before injection of [^{14}C]urea (kg.)	Urea contents (m-moles)	Treatment		Rate of urea breakdown (μmoles/hr.)	
			Before injection of [^{14}C]urea (*a*)	After 2 hr. (*b*)	After treatment (*a*)	After treatment (*b*)
4	2·70	12	None	Stomach removed	63	64
5	1·55	6·8	Intestine removed from pylorus to anus	Stomach removed	40	40
10	1·15	5·2	None	None	41	43
11	1·96	8·8	All abdominal viscera removed*	None	37	—
12	1·51	6·7	Kidneys, intestine from pylorus to anus, removed	None	24	27
13	1·50	6·7	None	Kidneys removed	56	58
14	1·4	6·4	Kidneys, uterus, ovaries, and gastro-intestinal tract, from cardiac sphincter to anus, removed	None	65	67
22	1·3	5·9	Gastro-intestinal tract, from cardiac sphincter to anus, removed	None	1	—
23	1·6	7·4	Gastro-intestinal tract, from cardiac sphincter to anus, removed	None	37	—
26	—	—	Blood supply left only to heart and lungs	None	$<0·5$	—
27	—	—	Blood supply left only through head and neck, heart and lungs	None	12	—

* These were the liver, stomach, intestine from pylorus to anus, spleen, pancreas, suprarenals, kidneys, bladder, uterus, and ovaries.

ranged from 0·011 to 0·61 % of the total ^{14}C injected/hr., showing that 1–67 μmoles of urea/hr. were hydrolysed in the body (Table 2).

The influence of the viscera on the breakdown of urea

When the stomach was removed 2 hr. after injection of the isotopic material, there was no decrease in the rate of expiration of [^{14}C]carbon dioxide over the next 2 hr. (Fig. 1, curve *A*). When this experiment was repeated on an animal from which the intestinal tract, from pyloric sphincter to anus, had been removed before the injection of the labelled urea, a similar result was obtained (Fig. 1, curve *B*). Previous experiments (Kornberg *et al.* 1952*a*) had established that cessation of [^{14}C]carbon dioxide production in the body would be followed by a rapid fall in the specific activity of expired carbon dioxide. These results therefore show that, although suspensions of the gastric mucosae of these three cats were found to have urease activity with Q_{urea} values of 2, 8 and 14, the breakdown of urea in these cats was not due to the activity of gastric urease. They further show (cf. Table 2) that this breakdown was also not due to the action of bacteria in the intestine, but was due to the

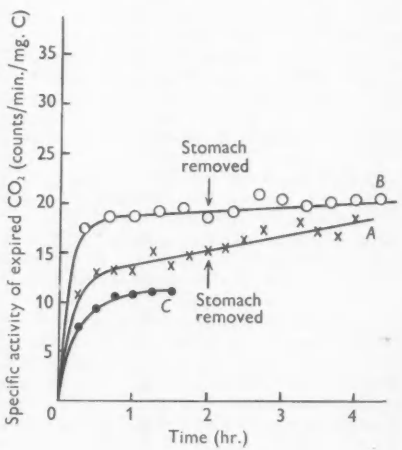

Fig. 1. The expiration of $^{14}CO_2$ after intravenous injection of [^{14}C]urea into fasting, anaesthetized cats with tied ureters. 2 130 000 counts/min. injected at 0 hr. *A*, cat 4; *B*, cat 5; intestine removed from pylorus to anus at 0 hr.; *C*, cat 11; all abdominal viscera removed at 0 hr. For further details, see Table 2.

occurrence of urea-splitting processes elsewhere in the body.

This failure to abolish the breakdown of urea by removal of the intestine (Fig. 1, curves *B* and *C*; Table 2) was again demonstrated when the gastro-intestinal tract, from cardiac sphincter to anus, was removed some hours after the injection of the urea, and only a slight decrease in the specific activity of the expired carbon dioxide occurred (cf. Kornberg *et al.* 1954). The observed breakdown of urea was therefore not due to the presence, throughout the experiments, of urea-splitting micro-organisms in this part of the intestinal tract, as had been suggested from earlier experiments on the metabolism of [^{15}N]urea in rats (Schoenheimer, 1942; Bloch, 1946).

Curve *C* of Fig. 1 was obtained with a cat (no. 11, Table 2) from which had been removed, before the injection of the urea, the liver, stomach, intestine from pylorus to anus, spleen, pancreas, suprarenals, kidneys, bladder, uterus, and ovaries. Despite this almost complete evisceration, the breakdown of urea occurred at a rate comparable with that found with normal cats, since 37 μmoles of urea were hydrolysed in the first hour. This showed that the organs removed made no major contribution to the breakdown of urea by the normal cat.

Three explanations appeared to be possible to account for this breakdown. (*a*) The rapid removal of ammonia from the blood by the liver might cause the reactions

$$CO(NH_2)_2 \rightleftharpoons NH_4^+ + OCN^- \xrightleftharpoons[-2H_2O]{\text{(cyanase?)}\ +2H_2O} NH_3 + HCO_3^- \rightleftharpoons \text{expired } CO_2$$

(NH_4^+ removed; NH_3 removed)

to move to the right, thus causing a non-enzymic breakdown of urea. (*b*) Urease might be distributed throughout the body but not localized in any one particular tissue. (*c*) Some tissue or tissues not yet identified might contain urease or urea-splitting organisms in quantities sufficient to catalyse the observed hydrolysis of urea.

Physicochemical transformations of urea

Urea in neutral, aqueous solution is in equilibrium chiefly with ammonium, cyanate, bicarbonate, and carbamate, ions. In the body, any ammonia thus arising from urea would be rapidly removed from solution, and any bicarbonate would mix with the total carbon dioxide of the body and would subsequently be expired. It was therefore conceivable that the small amounts of [^{14}C]carbon dioxide expired by cats after intravenous injection of [^{14}C]urea might arise from the disturbance in the body, by the two factors mentioned above, of the normal equilibrium between urea and its decomposition products. Reactions relevant to this physicochemical decomposition of urea (Wyatt & Kornberg, 1952) provided information from which were calculated (Kornberg, 1953) the amounts of [^{14}C]carbon dioxide which would arise by these non-enzymic mechanisms under the conditions existing in the cats. If the hydrolysis of the cyanate ion were not enzymically catalysed, the total quantity of [^{14}C]carbon dioxide produced by the physico-chemical decomposition of the injected [^{14}C]urea would be of the order of 0·004 μmoles/hr. instead of the average of 41 μmoles normally produced in that time. The rate of this non-enzymic breakdown of urea thus is too slow, by a factor of 10 000, to account for the observed rates of breakdown.

It has been suggested (Holtham & Schütz, 1948) that there is in blood an enzyme, cyanase, which catalyses the hydrolysis of cyanate. If such an enzyme were to play a part in the above scheme of conversion of urea into carbon dioxide, the rate calculated for this conversion would be increased at most by a factor of about 100, i.e. the total quantity of [^{14}C]carbon dioxide produced in this case would rise to a maximum of approximately 0·5 μmoles/hr., which is still only 1 % of the observed average rate.

These calculations therefore show that neither with nor without the intervention of a cyanase can the observed breakdown of urea in cats be due solely to the physicochemical reactions of urea under the present conditions, but that an enzymic cleavage of urea must occur.

The distribution of urease in the tissues

In order to establish the site of the enzyme responsible for the *in vivo* hydrolysis of urea, studies were made of the possible urease activity of various cat tissues.

Samples of brain, duodenum, ileum, kidney, liver, oesophagus, ovary, pituitary and adrenal glands, and skeletal muscle, were removed from cats and, with the previously described precautions against bacterial contamination, were ground with a little sand and twice their weight of water.

Samples of the resultant suspensions were tested for the presence of bacteria: growth of urea-splitting organisms occurred only from the suspensions of kidney, ileum, and oesophagus.

Samples of all tissue suspensions were placed in all-glass aeration units (Kornberg *et al.* 1953*a*), together with unlabelled urea containing added [^{14}C]urea (200 000 counts/min.), and sodium phosphate buffer, pH 7·4, both to final concentrations of 0·01 M. The units were placed in a water bath at 37° and a slow stream of 5 % CO_2 in O_2 was bubbled through the suspensions for five 1 hr. periods. The emergent CO_2 was collected in 3 N-NaOH and its specific activity assayed.

Although suspensions of gastric mucosa treated in this manner caused the rapid appearance of [^{14}C]carbon dioxide in the emergent gas, no such hydrolysis could be detected with any tissue tested except oesophagus and ileum: the rate of production of [^{14}C]carbon dioxide from the other tissue suspensions was immeasurably small, and the rate of urea breakdown was thus less than 0·0004 μmoles/hr. These results, together with those reported for the breakdown of urea in partially eviscerated cats, exclude the brain, duodenum, kidneys, liver, ovaries, pituitary and adrenal glands, and skeletal muscle, as the sites of the urease responsible for the *in vivo* hydrolysis of urea.

The localization of urease in the head and neck. The possibility that urea-splitting processes in the head and neck of the cats might be responsible for this hydrolysis of urea, was investigated after connecting the cats to a modification of the usual respiration circuit.

The tracheal cannula was connected to a valve, which in turn was connected both to a respiration pump and to the usual respiration circuit, in such a way that the lungs were inflated by the pump and deflated by the respiration circuit, and the chest could thus be opened without killing the cat. The gastro-intestinal tract from duodenum to rectum was removed in the first experiment, as were also the ovaries, uterus, spleen, pancreas and bladder. The oesophagus was removed from its junction with the mouth downwards to the cardiac sphincter. Ligatures were then tied round the carotid arteries and the jugular veins, three more were tied tightly round the neck, and the head and neck above the ligatures were cut off. The now headless cat had, after intravenous injection of dextran solution (6% (w/v) in 0·9% (w/v) NaCl), a blood pressure of 60 mm. Hg; it maintained this pressure and manifested strong, regular heart beats throughout the experiment. [^{14}C]Urea was injected intravenously and the expired air continuously collected in 15 min. samples over the next 2 hr. 15 min.

The total carbon dioxide expired over this time had a very low specific activity and represented a total of only 0·07 % of the total isotope injected. Analysis of the blood taken at the end of the experiment showed that the specific activity of the urea at this time was 18000 counts/min./mg. carbon; the low specific activity of the expired carbon dioxide was therefore due to a greatly reduced rate of urea breakdown in the cat. The rate observed, 6 μmoles/hr., was less than 15 % of the average noted in cats which had not been decapitated.

This experiment was repeated with a cat from which only the head was removed before injection of the isotopic material. The rate of urea breakdown was 4 μmoles/hr. and was unaffected by subsequent removal of the oesophagus, and of the gastro-intestinal tract. A third cat which, by accident, had not been fasted, was decapitated before injection of [^{14}C]urea. It hydrolysed 17 μmoles of urea in the first hour (Fig. 2), at which time the gut, from pyloric sphincter to anus, was removed. The amount of urea broken down over the next hour (15 μmoles) was not significantly lower, confirming that this hydrolysis was not caused by urease in the intestine. When the stomach was removed at 2 hr., the rate of expiration of [^{14}C]carbon dioxide fell rapidly, only 5 μmoles of urea being hydrolysed in the period of the experiment from 2 to 3 hr. Since the stomach contents were found to be strongly acidic and to consist of partly digested food, the high rates of breakdown noted in the early stages of the experiment were due to the activity of gastric urease concomitant with the secretion of gastric acid (cf. Kornberg & Davies, 1952*b*; Kornberg *et al.* 1954). The rapid fall in the rate of urea breakdown after gastrectomy showed that the residual urease activity in the cat was at most 10 % of that observed on the average in normal cats.

The ability of the head and neck regions to catalyse the breakdown of urea was demonstrated in an experiment in which the circulation to parts below the thorax was cut off.

Blood from the aorta was passed directly into the inferior vena cava through polythene tubing and an artificial resistance, attached to a mercury manometer, which served to regulate the blood pressure in this external circulation. Both the aorta and the inferior vena cava were tied below the cannulae inserted into them, thus leaving in the animal only the pulmonary circulation, and the systemic circulation to the head and neck.

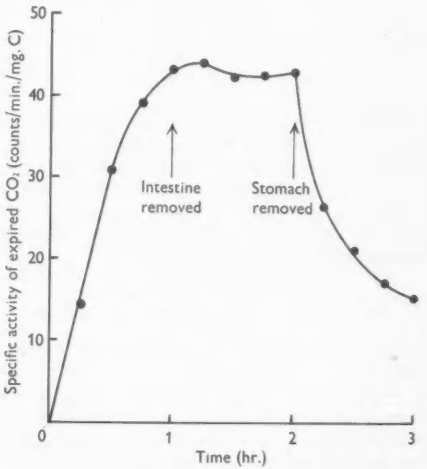

Fig. 2. Urea breakdown in an acid-secreting, decapitated cat. Female cat, 1·9 kg.; decapitated before injection of [^{14}C]urea (3500000 counts/min.) at 0 hr. Intestinal tract from pylorus to anus removed at 1 hr.; stomach removed at 2 hr. The stomach contents were strongly acidic and contained partly digested food.

When [^{14}C]urea was injected into this preparation, the usual rapid appearance of [^{14}C]carbon dioxide in the expired air occurred. Since the liver of this cat received no blood, no urea synthesis took place, and the concentration of urea in the blood remained constant. A total of 612 counts/min. as [^{14}C]carbon dioxide were liberated/hr. in the cat from [^{14}C]urea in the body fluids which had an activity of 360 counts/min./ml. water. The concentration of urea in the plasma was 7·1 mM. Hence the activity of the urea in the cat was 51 counts/min./μmole urea, and 12 μmoles of urea/hr. were therefore hydrolysed. This rate of breakdown was somewhat lower than the average normally observed in whole cats (Table 2), but was of the same order of magnitude, and higher than the breakdown occurring in decapitated animals.

These results thus show that nearly all the observed hydrolysis of urea in cats not secreting gastric juice occurs through the action of urease situated in the head and neck region of the animals.

Urease activity of cat gastric mucosa. Of thirteen cats examined, ten showed gastric urease activity *in vitro*, the Q_{urea} ranging from 2 to 41, average 14, a further cat examined had a Q_{urea} of 428, and two apparently normal cats showed no gastric urease activity ($Q_{urea} < 0{\cdot}1$). All suspensions manifesting urease activity also contained urea-splitting staphylococci (cf. Kornberg *et al.* 1954). The wide variation in the urease activity manifested *in vivo* by normal cats (Table 2), which would be expected as a consequence of a natural variation in the intensity of infection with urea-splitting micro-organisms, was therefore also seen in the wide variation in the urease activity of gastric mucosal suspensions.

The effect of an antibacterial mixture on the breakdown of urea

On the suggestion of Prof. A. Baird Hastings, of Harvard University, the effect on the urea breakdown of administering an antibacterial mixture was studied. Each dose of this mixture (which will for convenience be referred to as 'PTS mixture') consisted of 500 000 units of penicillin, injected intramuscularly as the sodium salt, and 500 mg. Terramycin (Pfizer Ltd., Folkestone) with 2·5 g. sulphaguanidine, administered orally.

When cats were treated with this 'PTS mixture' (two doses per day) for 4 days, and [^{14}C]urea was injected after this time, the carbon dioxide expired was of very low specific activity, and the total amounts of urea hydrolysed by two cats were only 1 and 3 μmoles/hr., respectively. Both the intestinal contents and the faeces were free of viable bacteria.

Manometric experiments showed that 'PTS mixture' at even 10 times the concentration given to the cats did not inhibit the urease activity of gastric mucosal suspensions. It was further shown (Kornberg *et al.* 1954) that the mixture was without direct effect on gastric urease in the intact cat, but acted by removing urea-splitting micro-organisms from the animals. These findings thus demonstrated that neither *in vitro* nor *in vivo* did the 'PTS mixture' inhibit urease. The abolition of the enzymic hydrolysis of urea in cats by treatment with 'PTS mixture' makes it highly probable that the enzyme responsible for the breakdown of urea in untreated cats is of bacterial origin.

DISCUSSION

The 'urea space' and total body water

Urea has been generally regarded as a substance which is uniformly distributed throughout the fluids of the body, although minor concentration differences have been reported in some tissues. Thus, Myers & Fine (1919), Cockrill (1931), Leopold & Bernhard (1917) and Fremont-Smith, Dailey, Merritt, Carroll & Thomas (1931), reported that the concentration of urea in the cerebrospinal fluid was lower than that of the plasma, and Ralls (1943) found a lower concentration in the blood cells. The concentration of urea in gastric juice is also lower than that in the plasma (Kornberg *et al.* 1954).

The error made in assuming urea to be freely diffusible and uniformly distributed throughout all the body water is, however, quantitatively insignificant, and urea has been used frequently in recent years for the measurement of total body water (Painter, 1940; Steffensen, 1947; McCance & Widdowson, 1951*a*, *b*; Eggleton, 1951). It was usually assumed in these experiments that the relatively large amounts of urea administered caused no diuresis and no changes either in the urea clearance or in the rate of formation of urea in the body, which might otherwise vitiate the results.

These assumptions became unnecessary when isotopic urea was used, since the urea was administered in negligibly small quantities. The 'urea space' by these means was found to be 64·5–65 % of the body weight (Kornberg *et al.* 1952*a*), which is in good agreement with the value of $63 \pm 1{\cdot}6$ % reported by Eggleton (1951), who did not use labelled urea. This showed that the assumptions made in the use of unlabelled urea were justified.

The total body water of cats, determined by complete desiccation of the animals, was first measured by Hatai (1917) and found to be 66·7 % of the body weight. Recent experiments with heavy water (Moore, 1946; Edelman, 1953) gave slightly lower results of 62–65 %. All these values are, however, in good agreement with the values found for the 'urea space' of the animals, and it must therefore be concluded that, within the limits of experimental error, the 'urea space' and the total body water are identical.

The in vivo *hydrolysis of urea*

When [^{14}C]urea was intravenously injected into cats, [^{14}C]carbon dioxide rapidly appeared in the expired air. The breakdown of urea thus observed varied greatly from cat to cat, but was constant within any one experiment (Table 2). In attempts to establish the site of this hydrolysis, most of the viscera were removed from cats without noticeably affecting the breakdown of urea in these animals. This ruled out any major concentration of urease in the liver and kidneys, as had been suggested by FitzGerald (1950). Subsequent experiments in which ground-up tissues were incubated with [^{14}C]-urea confirmed that no urea hydrolysis occurred in the muscles and these tissues, which represented the bulk of the viscera.

The removal of the stomach and intestine also had no effect on the breakdown of urea in these non-secreting cats. Since urea-splitting organisms could be isolated from both the stomach and intestine, it was apparent that the circulating urea did not come into effective contact with these sources of urease, nor with the gastric urease which could in most cases be shown to be present in the ground-up gastric mucosae. These findings are explained by the observation that clamping off the blood supply of the stomach (and therefore damaging the stomach) caused a manifestation of gastric urease activity *in vivo* (Kornberg *et al.* 1953*a*), and by the fact that the measured concentrations of urea plus half the ammonia in gastric juice, even with cats treated with 'PTS mixture' (which are devoid of gastric urease activity) was less than half those of the blood (Kornberg, 1953; Kornberg *et al.* 1954). There was thus a 'resistance' to the effective contact of urea in the body fluids and the site of gastric urease. This 'resistance' presumably operated also in reducing the contact of the intestinal flora and the circulating urea to quantitatively insignificant proportions.

When these animals were treated with 'PTS mixture' the enzymic breakdown of urea was abolished. As the 'PTS mixture' had no direct inhibitory action on gastric urease either *in vitro* or *in vivo* (Kornberg *et al.* 1954), it was therefore probable that the urease responsible for the hydrolysis of urea in untreated cats was of bacterial origin. This was supported by the fact that one cat (no. 22, Table 2) hydrolysed urea at only 1 μmole/hr., whilst others treated identically or even more drastically (nos. 5, 11, 14, 23) broke down urea at 37–67 μmoles/hr. This variation would be expected with a variation in the intensity of bacterial infestation. The stomach and intestine, which contain the largest proportion of the bacterial content of the cat, were ruled out as the site of this urease; the remaining bacterially infested sites were the mouth, trachea, nasopharynx, and oesophagus. Decapitated cats hydrolysed urea at less than 15 % of the rates observed on the average in normal animals, whilst the isolated head and neck of a cat could break down urea at a rate of the same order of magnitude as intact cats. These findings show that the site of the urease was in the head and neck.

The comments offered above on the 'resistance' between the circulatory urea and the urease in the gastric mucosa and intestinal tract apply also to the urease in the oesophagus, and, indeed, removal of the oesophagus did not cause any diminution of the rate of urea hydrolysis in the animal. The mouth, trachea, and nasopharynx, however, are richly supplied with urea-splitting micro-organisms, and it has been shown (Cary, 1946) that there is in the intact animal a continual process of hydrolysis of salivary urea. Moreover, the concentrations in the saliva of the urea plus half the ammonia, equal the concentration of urea in the plasma (Hench & Aldrich, 1922), which again shows that a continual splitting of urea takes place in the mouth.

It is therefore concluded that the breakdown of urea in cats not secreting gastric juice is due to the presence of urease of bacterial origin in the head and neck region of the animals, and is probably a result of the continual hydrolysis of urea in the saliva and other secretions of the mouth and respiratory tracts.

SUMMARY

1. When [^{14}C]urea was injected intravenously into anaesthetized cats not secreting gastric juice, whose ureters had been tied, the isotope equilibrated within 1 hr. with the urea of the body. This was found to be dissolved in a volume of water ('urea space') corresponding to an average of 65 % of the body weight and equal to the total body water of the cats.

2. Of the [^{14}C]urea injected, amounts were hydrolysed which varied widely between different cats but were constant within any one experiment. The amounts of urea broken down (average 41 μmoles/hr.) were measured from the quantities of [^{14}C]carbon dioxide produced in the body and subsequently expired.

3. It was calculated that this *in vivo* breakdown of about 0·5 %/hr. of the body urea could not be due to physicochemical transformations, but that enzymic cleavage of urea must occur.

4. Removal of the oesophagus or all the abdominal viscera did not diminish the breakdown of urea. Although suspensions of only stomach, ileum, and oesophagus, showed urease activity *in vitro*, the site of the urease responsible for the *in vivo* breakdown was not, therefore, located in the oesophagus or abdominal viscera.

5. Cats whose head and neck had been removed before the injection of [^{14}C]urea, hydrolysed urea at

less than 15% of the average rate normally observed, but the head and neck regions on their own could break down urea at higher rates. Washings of mouth, nasopharynx, and trachea, contained high urease activities.

6. Urease activity was abolished by treating cats with a mixture of penicillin, Terramycin, and sulphaguanidine. This mixture had no direct effect on urease *in vitro* or *in vivo*, but acted by removing urease-containing bacteria from the cats.

7. It is concluded that the enzymic breakdown of urea in cats not secreting gastric juice is caused by urease of bacterial origin, situated almost entirely in the head and neck.

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The Activity and Function of Gastric Urease in the Cat

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The presence of urease in suspensions of the gastric mucosae of dogs, cats, sheep, and bullocks, was first established by Luck (1924). Rigoni (1930), Martin (1932), Cardin (1933), Hollàn (1947), Fossel (1947) and Glick (1949) showed the enzyme to be present also in human stomachs. FitzGerald (1946) claimed to have found urease activity in the gastric mucosae of frogs, rats and pigs.

Although the enzyme occurs in the ground gastric mucosa of a variety of animal species, the evidence for the *in vivo* activity of gastric urease was slight and often contradictory, and its physiological role was still a matter for conjecture when the present series of investigations was begun. As shown

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previously in experiments with urea, labelled with ^{15}N or ^{14}C, in intact cats (Davies & Kornberg, 1950*b*; Kornberg & Davies, 1952*a*, *b*; Kornberg, Davies & Wood, 1954), urea is broken down *in vivo*, but the enzyme responsible for this hydrolysis in cats not secreting gastric juice is situated almost entirely in the head and neck, is of bacterial origin, and is independent of the presence of the stomach (Kornberg *et al.* 1954). Only when the stomach was stimulated to secrete gastric juice did gastric urease activity manifest itself (Kornberg & Davies, 1952*b*), and it was therefore concluded that the *in vivo* activity of the enzyme is closely related to the activity of the secreting stomach.

The work described in this paper was undertaken to throw light on the possible physiological role of the enzyme.

Part of this work has been communicated to the Biochemical Society (Kornberg, Davies & Wood, 1953).

EXPERIMENTAL

The general experimental techniques used in the treatment of cats, in the collection and analysis of respiratory, blood, and tissue, samples, and in the determination of radioactivity, were those of Kornberg, Davies & Wood (1952), Kornberg *et al.* (1954). The special procedures employed are described later. Urease activity was expressed as Q_{urea} (μl. CO_2 evolved/mg. dry wt./hr., where 22·26 μl. of CO_2 at s.t.p. are equivalent to 1 μmole of urea).

RESULTS

The effect of acid secretion on urea breakdown

The *in vivo* activity of gastric urease, which could not be demonstrated in cats not secreting gastric juice (Kornberg *et al.* 1954), became apparent when cats were stimulated to secrete gastric acid.

Histamine (15 μg./min. in 0·5 ml. 0·9% (w/v) NaCl) was infused into the right jugular vein of a cat, which had been injected with [^{14}C]urea 45 min. previously.

The rate of secretion rose within 1 hr. 15 min. from 0 to 0·24 ml. of juice/min.; it fell again to zero when the infusion of histamine was discontinued (Fig. 1, curve 5*B*). These changes were accompanied by changes in the specific activity of the expired carbon dioxide (Fig. 1, curve 5*A*). Some 15–20 min. after the start of the infusion, the specific activity rose sharply from the steady level of 60 counts/min./mg. carbon, to a peak (reached 15 min. after cessation of the histamine infusion) of 86 counts/min./mg. carbon. It then fell rapidly within the next 45 min. to 66 counts/min./mg. carbon. Despite the almost complete evisceration of the animal 30 min. later, the specific activity decreased only slightly over the next 1 hr. 30 min.

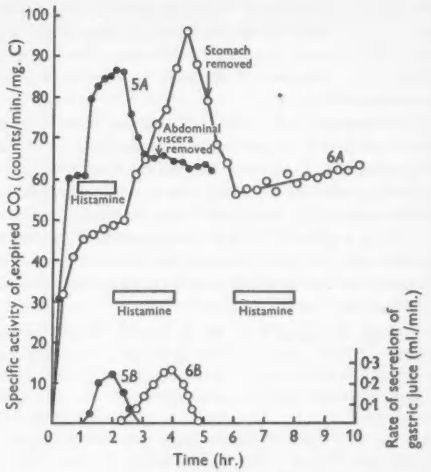

Fig. 1. The effect of infusions of histamine, removal of the stomach, and removal of abdominal viscera, on the specific activity of expired CO_2 and on the rate of secretion of gastric juice. [^{14}C]Urea injected at 0 hr. Cat 5: histamine infused from 0 hr. 45 min. to 2 hr., at 3 hr. 30 min. the gastro-intestinal tract (from cardiac sphincter to anus), spleen, pancreas, kidneys, and most of the liver were removed. Cat 6: histamine infused from 2 to 4 hr., stomach removed at 5 hr., histamine infused from 6 to 8 hr. For further details see text. Curves 5*A* and 6*A*: specific activity of expired CO_2. Curves 5*B* and 6*B*: rate of secretion of gastric juice.

These results show that the breakdown of urea by the cat was greatly accelerated by the administration of histamine, and that the rate of this increased breakdown was paralleled by the rate of secretion of acid juice. The failure of the specific activity to fall to any major extent after the almost complete evisceration of the animal confirms that most of the abdominal viscera do not participate in the hydrolysis of urea by cats not secreting gastric juice (Kornberg *et al.* 1954).

Similar results were obtained in a more extended experiment (Fig. 1, curves 6*A*, *B*). The infusion of histamine was begun 2 hr. after injection of [^{14}C]-urea and was maintained for 2 hr. During this time, 19·7 ml. of gastric juice were secreted, and the rate of secretion of juice (Fig. 1, curve 6*B*) again paralleled the rate of increased urea breakdown (Fig. 1, curve 6*A*). The specific activity of the expired carbon dioxide rose from 50 counts/min./mg. carbon at 2 hr. 15 min. to 96 counts/min./mg. carbon at 4 hr. 15 min. and, in the 2 hr. after cessation of the histamine administration, fell to 56 counts/min./mg. carbon. At 5 hr., the stomach of the cat was removed. From 6 to 8 hr., histamine was again infused into the now gastrectomized animal. There

was no major change in the specific activity of the expired carbon dioxide during or after the infusion, the curve of specific activity against time being of constant slope and apparently a continuation of the curve obtained in the first 2 hr. after injection of [^{14}C]urea.

These results confirm that the increased breakdown of urea observed was caused by the action of histamine, and that the site of this action was the stomach. The continued expiration of [^{14}C]carbon dioxide after removal of the stomach again proved the existence of urea-splitting processes in the body elsewhere than in the stomach (Langham, 1949; Kornberg *et al.* 1954).

The effect of the presence of acid in the stomach on the breakdown of urea

It has been suggested frequently that gastric urease, by producing ammonia from the hydrolysis of urea, may act as a neutralizing agent and protect the mucosa from attack by acid and pepsin (Luck & Seth, 1925; Linderstrøm-Lang & Ohlsen, 1936; Ohlsen, 1941; FitzGerald, 1946; Fossel, 1947; FitzGerald & Murphy, 1950; Glick, Zak & Von Korff, 1950; Von Korff, Ferguson & Glick, 1951). If this were indeed the physiological function of the enzyme, gastric urease activity might be expected to manifest itself not only during the secretion of acid juice, but also in response to the presence of acid in the stomach (FitzGerald & Murphy, 1950). The result of an experiment to test this point is shown in Fig. 2. [^{14}C]Urea was injected into a cat from which had been removed the intestinal tract from pylorus to anus, the pancreas, ovaries, uterus and both kidneys. The specific activity of the expired carbon dioxide reached a constant level of 16 counts/min./mg. carbon after 1 hr. At 2 hr. 20 ml. of 0·1 N hydrochloric acid were put into the stomach through a pyloric cannula. The acid remained in the stomach for 1 hr. Its presence did not cause any change in the specific activity of the expired carbon dioxide. When the acid was removed and the stomach was washed out with 0·9 % (w/v) sodium chloride, a small secretion of acid juice occurred, probably owing to the distension of the mucosa in the washing procedure. At the same time there was a slight rise in the specific activity of the expired carbon dioxide. At 3 hr. 45 min. a rapid secretion of acid was stimulated by the intravenous infusion of 8·3 mg. Priscol (2-benzylglyoxaline hydrochloride) in 4·88 ml. 0·9 % (w/v) sodium chloride in 15 min. There was a sharp rise in the specific activity of expired carbon dioxide when acid secretion commenced, and the curves of this increased urea breakdown (Fig. 2, curve *A*) and of the secretion of acid juice (Fig. 2, curve *B*) were again of the same shape. This showed that gastric urease was present and that its activity manifested itself *in vivo* not in response to the presence of acid, but as a consequence of the active secretion of acid juice by the stomach.

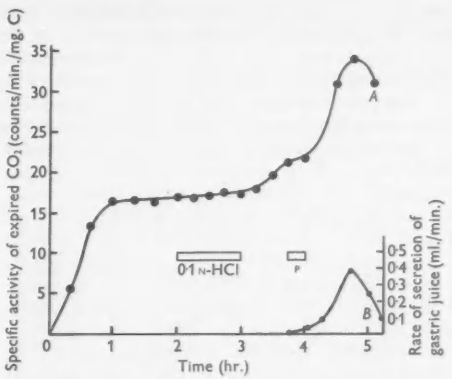

Fig. 2. The effect of the presence of acid in the stomach, and of an infusion of Priscol, on the specific activity of expired CO_2 and on the rate of secretion of gastric juice. Cat 7: kidneys, pancreas, intestine from pylorus to anus, removed before injection of [^{14}C]urea at 0 hr., 0·1 N-HCl (20 ml.) put into stomach from 2 to 3 hr., Priscol (P) infused from 3 hr. 45 min. to 4 hr. For further details see text. Curve *A*: specific activity of expired CO_2. Curve *B*: rate of secretion of gastric juice.

The effect of variations of blood flow through the stomach on the breakdown of urea

The results obtained on the breakdown of urea through the action of gastric urease suggested that the *in vivo* activity of the enzyme was closely linked with the activity of the secreting stomach. It was feasible that this enzyme activity was a consequence of the greatly increased blood flow through the stomach during the secretion of gastric juice (Cutting, Dodds, Noble & Williams, 1937*a*, *b*). This was investigated by varying the temperature of, and thus the blood flow through, the stomach of a cat (Cutting *et al.* 1937*b*). Warm water (41°) was circulated through a thin rubber balloon placed in the stomach, 2 hr. after injection of [^{14}C]urea. At 3 hr. cool water (31°) was substituted for the warm water. The pH of the surface of the mucosa remained neutral throughout the experiment. At 4 hr. the rubber balloon was removed, but it was not possible to stimulate acid secretion as the cat died shortly afterwards.

The effect on the expiration of [^{14}C]carbon dioxide of these variations in the rate of blood flow through the stomach is shown in Fig. 3. The circulation of warm water through the stomach was accompanied by a rise in both the rate of expiration of carbon dioxide-carbon (curve *B*) and of ^{14}C (curve *A*); the

circulation of cool water was accompanied by a fall in both these rates. The specific activity of the expired carbon dioxide (curve *C*) remained constant throughout the experiment and was not affected by the changes in the temperature of the stomach. This showed that the changes in the rates of expiration of both labelled and unlabelled carbon dioxide-carbon were due to changes in the metabolic activity of the cat, and were not due to the activity of gastric urease. The activity of this enzyme was not, therefore, governed solely by the blood flow through the stomach, but by some other factors related to the elaboration of gastric juice.

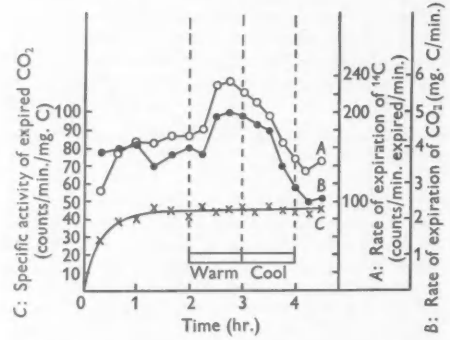
C: Specific activity of expired CO2 (counts/min./mg. C)
A: Rate of expiration of 14C (counts/min. expired/min.)
B: Rate of expiration of CO2 (mg. C/min.)
Warm
Cool
Time (hr.)

Fig. 3. The effect of variations in the blood flow through the stomach on the expiration of $^{14}CO_2$. Cat 8: [^{14}C]urea injected at 0 hr., water at 41° circulated through a rubber balloon, placed in the lumen of the stomach, from 2 to 3 hr.; water at 31° circulated from 3 to 4 hr. For further details see text.

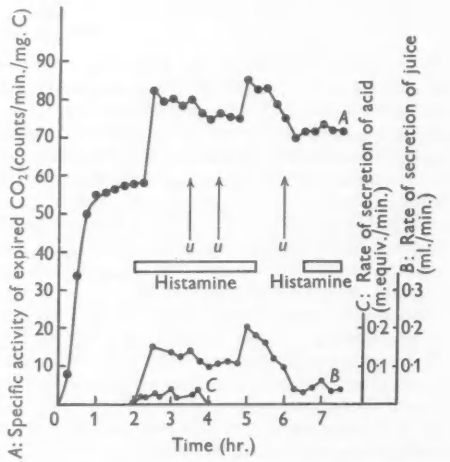
A: Specific activity of expired CO2 (counts/min./mg. C)
C: Rate of secretion of acid (m.equiv./min.)
B: Rate of secretion of juice (ml./min.)
Histamine
Histamine
Time (hr.)

Fig. 4. The effect of histamine and urogastrone on the secretion of gastric juice and on the expiration of $^{14}CO_2$. Cat 9: ovaries and kidneys removed before injection of [^{14}C]urea at 0 hr. Histamine infused from 2 hr. to 5 hr. 15 min. and from 6 hr. 30 min. to 7 hr. 30 min. Urogastrone (*U*) injected at arrows. For further details see text.

The effect of the secretion of non-acid juice on the breakdown of urea

Since the presence of acid in the stomach was not sufficient to evoke gastric urease activity, it was possible that the observed increased breakdown of urea was due, not to the secretion of gastric acid, but to the passage through the mucosa of urea-containing water in the secreted juice. This possibility was suggested by the result of an experiment in which a cat was stimulated to secrete acid gastric juice, by intravenous infusion of histamine, and in which urogastrone inhibited the secretion of acid but not the secretion of juice (Fig. 4). The rise in the specific activity of the expired carbon dioxide (*A*) was again paralleled by the rate of secretion of acid (*C*). After 3 hr. 30 min., 2 mg. of a urogastrone preparation, prepared according to Gray, Wieczorowski, Wells & Harris (1942), were injected, a further 2 mg. being given 45 min. later. The effect of these quantities of urogastrone was to abolish the secretion of titrable acid, but, unexpectedly, to leave the secretion of juice (*B*) unaffected. The specific activity of the expired carbon dioxide did not fall with the fall in the acid content of the juice, but remained high and in its variations with time reflected the rate of secretion of the now neutral juice.

At 5 hr. 15 min. the infusion of histamine was discontinued. Both the specific activity and the rate of secretion of juice fell. After a further injection of 4 mg. of the urogastrone preparation, neutral juice secretion recommenced and the specific activity of the expired carbon dioxide rose again, to remain constant despite a further infusion of histamine.

This experiment showed that the activity *in vivo* of gastric urease was associated with the passage of liquid from the blood to the lumen of the stomach, and was not dependent on the presence of acid in such a secretion.

The breakdown of urea associated with the passage of urea solution from the lumen of the stomach to the blood

Confirmation of these findings were obtained in two experiments in which [^{14}C]urea solution was made to pass through the gastric mucosa, from lumen to blood.

[^{14}C]Urea solution, containing 5 mM unlabelled urea, was placed in the cat's stomach, which had been ligated at the cardiac and pyloric ends. The pressure of liquid in the stomach was regulated by the height of a funnel containing

part of the urea solution which was connected by flexible tubing to a cannula inserted into the lesser curvature. In Expt. 20 (Fig. 5), the stomach was filled with the urea solution at zero time, the height of liquid in the funnel being adjusted to be level with the top of the stomach, i.e. the pressure of liquid in the lumen was 0–3 cm. of solution.

[^{14}C]Carbon dioxide quickly appeared in the expired air, the specific activity rising rapidly for 30 min. and more slowly after this time. The funnel was raised at 30 min. and more solution was added until the pressure at the top surface of the now greatly distended stomach was 13 cm. of solution. Owing to the corrugated nature of the mucosa, this distension of the stomach probably caused no major increase in the surface area of the mucosa available to the solution. This increase in the pressure, and therefore in the rate of passage of urea solution out of the stomach into the blood (cf. Davies & Terner, 1949), was accompanied by a rise in the specific activity of the expired carbon dioxide. At 1 hr., most of the urea solution was drained off and the pressure of the residual liquid in the stomach was again adjusted to 0–3 cm. of solution. This fall in the pressure, and therefore in the rate of flow of solution through the stomach wall, was reflected by a fall in the specific activity of the expired carbon dioxide. At 1 hr. 30 min., the pressure was again raised to 13 cm. above the top surface and the specific activity again rose sharply, reaching the value of 1540 counts/min./mg. carbon at 2 hr. 30 min., at which time the experiment was discontinued.

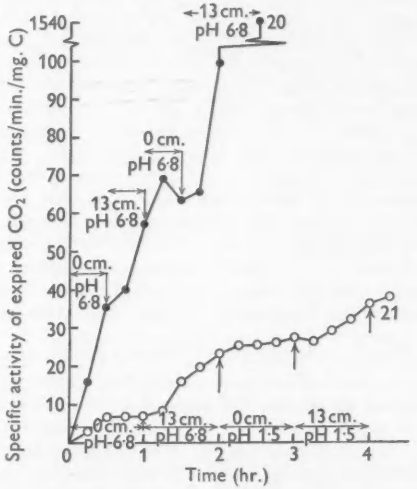

Fig. 5. The effect of changes in the pressure of [^{14}C]urea solution in the stomach on the specific activity of expired CO_2. Urea solution (5 mM), containing [^{14}C]urea, was put into the stomachs of cats 20 and 21 at 0 hr. The pH, and the pressures (in cm. of solution above the top surface of the stomach), are given on the figures. For further details see text.

Analysis of the acid-volatile [^{14}C]carbon dioxide of the urea solution removed from the stomach showed that less than 0·08% of the total radioactivity of the solution was present in this form, i.e. that the specific activity of the [^{14}C]bicarbonate present in the solution was less than 50 counts/min./mg. carbon: the very high specific activity observed at the end of the experiment in the carbon dioxide expired by the cat could not, therefore, be due to the passage into the blood of [^{14}C]bicarbonate produced by any bacterial breakdown of the urea solution in the lumen of the stomach. The specific activity of the urea in the blood at the end of the experiment was 42 counts/min./mg. carbon, which was approximately 40 times less than the specific activity of the acid-volatile carbon dioxide of the blood at this time.

These results showed that the rate of breakdown of urea by gastric urease could be determined by the rate of passage of urea-containing liquid through the mucosa, and that most of the urea thus passing through the stomach wall was hydrolysed before entering the blood; the urease responsible for this hydrolysis must therefore be located within the gastric epithelium.

A second experiment (Fig. 5, curve 21) gave similar results. After putting [^{14}C]urea solution at 0–3 cm. pressure, and pH 6·8 into the stomach, [^{14}C]carbon dioxide appeared rapidly in the expired air, the specific activity reaching a constant value after 30 min. At 1 hr., the stomach was distended by raising the pressure within it, to 13 cm. above the top surface; the increased flow of urea solution thus achieved was again accompanied by a rise in the specific activity of expired carbon dioxide. At 2 hr., all the urea solution was removed from the stomach and acidified to pH 1·5. The acid liquid was reintroduced into the stomach and maintained at 0–3 cm. pressure for 1 hr. The specific activity during this time continued to rise slightly since all the [^{14}C]carbon dioxide produced within the cat in the previous hour had not yet been expired (cf. Kornberg & Krebs, 1952), but the reduced rate of elimination of [^{14}C]carbon dioxide showed again that the lower rate of flow of urea solution across the mucosa was accompanied by a lower rate of urea breakdown. When the pressure was raised to 13 cm. above the top surface of the stomach at 3 hr., the increase in the rate of flow was again followed by a rise in the specific activity of expired carbon dioxide.

These results thus confirm that gastric urease activity *in vivo* can be evoked by a flow of urea solution through the stomach wall, the rate of urea breakdown depending on the rate of flow of solution

past the enzyme. Since there was no apparent difference in the response of the enzyme to urea solutions at pH 6·8 and at pH 1·5, it must further be concluded that (as already shown by the experiment with a cat secreting non-acid juice) the presence of acid in the liquid is not essential to the activity of the enzyme, and further that the urease must be situated in the mucosa at a site protected from contact with acid, which would inactivate the enzyme (cf. Hollàn, 1947).

The effect of antibacterial substances on urea breakdown

It has been shown previously (Kornberg *et al.* 1954) that the general enzymic breakdown of urea by cats not secreting gastric juice is abolished by administration of a mixture of penicillin, Terramycin, and sulphaguanidine ('PTS mixture'). (The composition of this mixture and its suggested mode of usage were kindly supplied by Drs A. Baird Hastings and R. Dintzis, of Harvard University.) When [^{14}C]urea was injected into cats, treated for 4 days previously with the 'PTS mixture', only traces of [^{14}C]carbon dioxide were expired, the amount of ^{14}C expired/hr. being but 0·010–0·015 % of the isotope injected. The rate of hydrolysis of urea in these cats was 1–3 μmoles/hr., which was less than 7 % of the average rate observed in untreated cats. When histamine was infused intravenously, acid gastric juice was secreted as usual, but there was no increase in the specific activity of expired carbon dioxide. The gastric mucosae of the treated cats were free from urease activity and viable bacteria.

The deposition of urease in the stomach

In eleven out of thirteen experiments on cats not treated with 'PTS mixture', aqueous suspensions of the gastric mucosae were found to possess urease activity. Bacteriological examination of such suspensions showed, however, that urea-splitting staphylococci were present in these suspensions, and, although in many cases the growth of organisms cultured from the mucosal suspensions was but slight, it was feasible that the gastric urease activity of secreting cats was due to the breakdown of urea by bacteria infesting the lumen of the stomach, and present throughout the experiments. Attempts were therefore made to grow bacteria, isolated from mucosal suspensions, at acid pH: it was found that no growth occurred at pH less than 4 and, further, that the addition of acid (final pH of 2) to suspensions of bacteria hydrolysing urea in manometric experiments caused an immediate loss of urease activity. It was already known (cf. Fig. 4, curve 21) that the urease in the mucosa was located at a site protected from destruction by acid. These considerations therefore made it unlikely that the urease activity of the stomach was due to the presence of bacteria in the lumen throughout the experiments, and suggested a deposition of urease by the bacteria on to the mucosal tissue as a more likely alternative.

Manometric investigations of the urease activities manifested by cultures of bacteria isolated from mucosal suspensions (Kornberg, 1953) showed that, after removal of the organisms grown in various nutrient media, the residual media contained urease, and that with increasing age of the cultures increasing amounts of urease appeared in the media. Whereas these findings do not necessarily show that the bacteria in the stomach are able actively to secrete urease, they support the view that they can deposit urease into the surrounding medium. This was further tested by treating a cat for 3 days with 'PTS mixture' (by which time the faeces contained no viable bacteria) and then removing a portion of the stomach from the corpus region. Analysis of the extirpated portion for urease activity and bacterial content showed that, although no viable bacteria were present, the mucosal suspension had high urease activity ($Q_{urea} = 23$). This demonstrated that gastric urease activity could, under these conditions, be present in the absence of viable organisms. When the cat had been given time to recover from the operation (10 days), treatment with 'PTS mixture' was recommenced and continued for 5 days. After this time the stomach was completely removed and analysed for urease activity and bacterial content. It was found that no viable bacteria were present and that all urease activity had disappeared. These results show that the abolition of urease activity in cats treated with 'PTS mixture' was not due to the inhibition of the enzyme by the mixture (Kornberg *et al.* 1954), but that it was due to the elimination of urea-splitting organisms. Since gastric urease could be detected after 3, but not after 5 days of treatment, it also seemed likely that the urease activity observed after the shorter time was due to the presence of enzyme of bacterial origin, which had been deposited in the mucosa but which had not yet been broken down by the cat. After the longer time, all the deposited enzyme had been destroyed.

The urea and ammonia contents of gastric juice

The concentration of urea in the gastric juice secreted by the cats was measured manometrically with jack bean urease (cf. Davies & Kornberg, 1951), and the ammonia content was measured by the method of Conway (1947). It was found that cats not treated with 'PTS mixture' secreted juice containing 0·51–0·67 mM urea, average 0·60 mM (eight samples). The ammonia concentrations ranged from 3·6 to 4·4 mM, average 4·2 mM, in good agreement with the average of 3·9 mM reported by Teorell (1933). Cats treated with 'PTS mixture' (whose stomachs contained no gastric urease)

Table 1. *The breakdown of* [^{14}C]*urea during the secretion of gastric juice*

(The average urea concentration in the body fluids of the cats was taken to be 7 mM. The amounts of $^{14}CO_2$ expired as the consequence of gastric secretion were the amounts observed minus the amounts which would have been expired had secretion not occurred: these quantities were measured from the graphs of time against percentage of injected ^{14}C expired. The urea space was taken to be 65% of the body weight (Kornberg *et al.* 1954).)

Cat no.	Body wt. (kg.)	Urea space (ml.)	Urea content of cat (m-moles)	Extra $^{14}CO_2$ expired during secretion (% of injected ^{14}C)	^{14}C retained by cat (% of injected ^{14}C)	Duration of secretion (hr.)	Extra [^{14}C]urea hydrolysed during secretion (% of injected ^{14}C)	Extra [^{14}C]urea hydrolysed during secretion (μmoles)	Rate of hydrolysis of urea (μmoles/hr.)
4	1·53	975	6·82	0·37	43	2	0·65	45	23
6	1·15	750	5·25	0·50	43	2	0·88	46	23
7	1·70	1110	7·77	0·28	43	2	0·49	38	19
9	1·42	925	6·48	0·62	32	5	0·91	59	12

secreted juice containing 2·6–3·4 mM urea, average 3·0 mM (four samples) and no detectable ammonia, i.e. less than 0·05 mM.

Quantitative aspects of gastric urease activity

The extra expiration of [^{14}C]*carbon dioxide during secretion.* In the experiments with acid-secreting cats not treated with 'PTS mixture' the activity of gastric urease was observed by the expiration, above the 'resting level', of increased amounts of [^{14}C]-carbon dioxide. The total quantity of extra [^{14}C]-carbon dioxide thus expired was related to the quantity of extra [^{14}C]urea broken down through the action of gastric urease; no direct calculation of this amount of urea hydrolysed could be made, however, until the amounts of [^{14}C]carbon dioxide expired had been corrected for the amounts retained by the cats during the relevant periods as a consequence of the exchanges of the isotopic material in the body (cf. Kornberg *et al.* 1952; Kornberg & Krebs, 1952).

Experiments on the metabolism of [^{14}C]bicarbonate intravenously injected into cats had shown that the rate of elimination of ^{14}C from bicarbonate was given by the relation

$$(100-P) = 28{\cdot}4e^{-0{\cdot}243t} + 41{\cdot}5e^{-0{\cdot}0234t} + 30{\cdot}2e^{-0{\cdot}00119t},$$

where P is the percentage of the injected ^{14}C expired t min. after the injection (Kornberg *et al.* 1952). From this it was calculated (Kornberg, 1953) that the percentage P' of the total [^{14}C]carbon dioxide produced through the action of gastric urease, which would be retained in the cat t' min. after the commencement of urease activity, was given by the relation

$$P' = 1/t' \,[117(1-e^{-0{\cdot}243t'}) + 1770\,(1-e^{-0{\cdot}0234t'}) + 25400\,(1-e^{-0{\cdot}00119t'})].$$

Substitution of the appropriate times t' (the durations of the secretory periods for the four cats studied) in this equation yielded values for the amounts of [^{14}C]carbon dioxide produced which were not expired but retained by the cats; these values are shown in column 6 of Table 1. The graphically evaluated quantities of extra [^{14}C]-carbon dioxide expired during the activity of gastric urease were therefore corrected to give the total quantities (expressed as percentages of the total isotope injected as [^{14}C]urea) of extra [^{14}C]-carbon dioxide produced during the secretory periods.

Approximate values in m-moles of these extra quantities of urea hydrolysed were derived by taking the average urea concentration in the body fluids of the cats to be 7 mM, and thus by expressing the percentages of urea hydrolysed in terms of the total amounts of urea in the cats. These values are given in the last column of Table 1. The quantities of urea hydrolysed through the action of gastric urease were 12–23 μmoles of urea/hr. It has been stated (FitzGerald, 1946) that sufficient urease can be extracted from a cat's stomach to produce 66 mg. ammonia nitrogen from the hydrolysis of urea/hr., i.e. to hydrolyse 2·9 m-moles of urea/hr. However, the *in vivo* breakdown of urea by gastric urease proceeds at less than 1% of this rate (cf. Kornberg & Davies, 1952*a*).

The disappearance of urea from the gastric juice. Experiments with [^{15}N]urea by Von Korff *et al.* (1951) showed that the isotope abundance ratio of the ammonia nitrogen of canine gastric juice was identical with that of the blood-urea nitrogen, proving that the ammonia of the juice was exclusively formed from blood urea. It is thus possible to calculate the amounts of urea hydrolysed by gastric urease in the present experiments. In cats not treated with 'PTS mixture', the sum of urea nitrogen plus half the ammonia nitrogen of the juice averaged 3·2 mM; in cats treated with the mixture, this figure averaged 3·0 mM. It was therefore assumed that the urea concentration of the secreted juice would be 3·0 mM in the absence of gastric urease activity, and that the final concentration of urea of the juice was the average concentration determined in previous experiments, 0·60 mM. Hence the amounts of urea hydrolysed through the action of gastric urease were taken to be

Table 2. *The hydrolysis of urea during the secretion of gastric juice*

(The initial concentration of urea in the secreted juice was assumed to be the sum of the observed concentrations of the urea + half the ammonia, i.e. 3·0 mM. The final urea concentration was taken to be the average determined, 0·60 mM. Hence the amount of urea hydrolysed by gastric urease was taken to be 2·4 m-moles/l. of secreted juice.)

Cat no.	Vol. of juice secreted (ml.)	Total amount of urea hydrolysed (μmoles)	Duration of secretion (hr.)	Rate of hydrolysis (μmoles/hr.)
4	17·7	43	2	22
6	19·7	47	2	23
7	15·8	38	2	19
9	25·0	60	5	12

2·4 m-moles/l. of secreted juice. From this figure, the amounts of urea broken down were calculated to be 12–23 μmoles/hr., in agreement with the quantities calculated to be hydrolysed from the expiration of [^{14}C]carbon dioxide (Table 2). These calculations thus show that the extra [^{14}C]carbon dioxide output concomitant with the secretion of gastric juice was stoicheiometrically related to the quantities of urea hydrolysed through the action of gastric urease, and confirm the findings of Von Korff *et al.* (1951) that the ammonia, set free in the gastric mucosa through the action of the enzyme, migrates predominantly into the lumen of the stomach, whilst the carbon dioxide similarly liberated appears predominantly in the blood.

DISCUSSION

The evidence for the activity of gastric urease in vivo

Although the presence of gastric urease in suspensions of the gastric mucosae of a wide variety of animal species was firmly established, the evidence for the *in vivo* activity of the enzyme was slight and often contradictory. Luck & Seth (1925) reported that, after administering urea solutions to anaesthetized dogs (by stomach tube or by intravenous injection), the ammonia content of the blood entering the stomach was lower than that leaving it. This could not be confirmed by Mann, Grindlay & Mann (1941), who described this blood ammonia as an artifact. Rigoni (1931) and Sumida (1935) perfused isolated dog stomachs with saline solutions containing urea, and stated that the emergent fluid contained less urea and more ammonia than the initial perfusing liquid, thus showing that urea had been hydrolysed in the stomach. Muraoka (1935) and Kitsukawa (1936), who perfused isolated dog stomachs with ammonia and either arginine or ornithine plus citrulline, claimed, however, that small quantities of urea were synthesized in their experiments. Since these claims are contradictory to the earlier ones, the result of the perfusion experiments must be regarded with caution.

A threefold increase in the ammonia concentration in histamine-stimulated dog gastric juice after intravenous injection of urea, was reported by Vladesco, Simci & Popesco (1931). There was, however, a concomitant threefold decrease in the volumes of juice secreted in these experiments; the conclusion that breakdown of urea in the stomach had occurred is therefore not necessarily warranted. More convincing was the work of Hessel, Pekelis & Meltzer (1933), who showed that in nephrectomized dogs the rise in the ammonia content of the gastric juice matched the rise in the concentration of blood urea.

Recent work with labelled urea showed that urea is hydrolysed in the body (Leifer, Roth & Hempelmann, 1948; Langham, 1949; Jones, 1950; Davies & Kornberg, 1950*b*; Hastings, Langham, Carter & Roth, 1950; Skipper *et al.* 1951; Kornberg & Davies, 1952*a*, *b*), but that this breakdown is independent of the presence of the stomach in cats not secreting gastric juice (Kornberg & Davies, 1952*b*; Kornberg *et al.* 1954). The experiments reported in the present paper show that the activity of gastric urease in the cat is a consequence of the passage of urea-containing liquid through the gastric mucosa, and that the presence of acid in such a liquid is not essential to the activity of the enzyme. The abolition of all gastric urease activity after treatment of cats with 'PTS mixture' further makes it highly probable that gastric urease is of bacterial origin.

The physiological role of gastric urease

The physiological role of gastric urease has been a matter for considerable speculation. After Mathews (1925) suggested that the hydrogen ions of the gastric acid were derived from ammonium ions, by the reaction $NH_4Cl \rightarrow NH_3$ (absorbed) + HCl, it was thought that the function of gastric urease was to provide ammonium ions for this reaction by the hydrolysis of urea to ammonium carbonate (Rigoni, 1930; Martin, 1932; Cardin, 1933; Mann & Mann, 1939; Martinson, 1950). A tentative hypothesis, somewhat similar to that of Mathews (1925), was put forward by Glick (1949). Studies on the isolated gastric mucosa of *Rana temporaria* showed, however, that the rate of secretion of acid can be at least 500 times greater than the rate of urea breakdown, and that the store of urea in the isolated stomach is less than 3 % of the amount required to account for the acid secreted (Davies & Kornberg, 1950*a*, 1951). The work reported in the present paper also shows that the rates of acid secretion in the cat are greatly in excess of the rates of urea breakdown through the action of gastric urease, and it must therefore be concluded that gastric urease plays no part in the mechanism of acid secretion by the stomach. This view has been accepted by Glick and his co-workers (Glick *et al.* 1950).

It has frequently been suggested that gastric urease, by hydrolysing urea to ammonium carbonate, provides base which can neutralize part of the gastric acid, and that the enzyme therefore acts as an agent protecting the mucosa from attack by acid and pepsin (Luck & Seth, 1925; Linderstrøm-Lang & Ohlsen, 1936; Ohlsen, 1941; FitzGerald, 1946; Fossel, 1947; FitzGerald & Murphy, 1950; Glick *et al.* 1950; Von Korff *et al.* 1951). The present results show, however, that the activity of gastric urease is not elicited by the presence of acid in the stomach and is not dependent on the secretion of an acid juice. Further, since apparently normal cats, devoid of gastric urease activity (Kornberg *et al.* 1954), and cats treated with 'PTS mixture', secreted acid without causing damage to the stomach, it would seem that the small neutralization of acid (<2%) through the action of gastric urease is not essential to the welfare of the stomach. It is therefore concluded that gastric urease plays no essential role in gastric physiology.

SUMMARY

1. The *in vivo* hydrolysis of urea was determined from the expiration of [^{14}C]carbon dioxide after intravenous injection of [^{14}C]urea into fasted, anaesthetized cats, whose ureters were tied. When cats secreted gastric acid, there was an increase in the rate of urea breakdown, due to the activity of gastric urease. The gastric juice secreted contained, on average, 0·60 mM urea and 4·2 mM ammonia, and suspensions of the gastric mucosae contained urea-splitting staphylococci.

2. The activity of gastric urease could not be elicited by the presence of acid in, nor by variations in the blood flow through, the stomach. It was, however, evoked when the stomach secreted a non-acid juice.

3. By forcing [^{14}C]urea solution at pH 1·5 or pH 6·8 through the gastric mucosa from stomach lumen to blood, it was shown that the activity of gastric urease was dependent upon the flow of urea-containing liquid past the enzyme, which is located in the stomach at sites protected from inactivation by acid.

4. The enzymic hydrolysis of [^{14}C]urea was abolished by treating cats for 4 days with a mixture of penicillin, Terramycin and sulphaguanidine, which was shown not to inhibit gastric urease either *in vitro* or *in vivo* but to act by removing urea-splitting bacteria from the cats. These cats were free of gastric urease activity, and secreted gastric juice containing, on average, 3·0 mM urea and no detectable (<0·05 mM) ammonia. This confirms that the ammonia of the gastric juice was derived from the hydrolysis of urea by gastric urease.

5. From the quantities of [^{14}C]carbon dioxide liberated into the blood, and the ammonia secreted in the gastric juice, it was calculated that gastric urease hydrolysed 12–23 μmoles of urea/hr., i.e. about 0·2%/hr. of the total urea in the cat. The ammonia thus produced could neutralize less than 2% of the acid secreted during this time.

6. The role of urease in the stomach is discussed. It is concluded that gastric urease is of bacterial origin, and plays no essential role in the physiology of the stomach.

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Changes in Lens During the Formation of X-ray Cataract in Rabbits. 2

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There is progressive and finally almost complete loss of glutathione and of protein sulphydryl groups during the formation of lens opacity. This has long been known and has led to many investigations to determine at what stage the change in the sulphydryl groups takes place. Bellows & Rosner (1937) showed that the glutathione decreased before any opacity developed in the lenses of rats fed with galactose, and Bellows & Schoch (1950) observed the same in rabbits after injection of alloxan. Kinsey & Merriam (1950) measured glutathione synthesis by culturing lenses *in vitro* in the presence of [C^{14}]glycine, and in a small series they found that such lenses, from rabbits that had been fed on naphthalene 2–5 days previously, synthesized less glutathione than lenses from normal rabbits. Twelve days after the last naphthalene dose, the rate of glutathione synthesis had returned to normal. Kinsey & Merriam therefore considered that during the development of cataract the synthesis of glutathione became impaired, but that it might recover.

The earliest change which we have found in the rabbit lens during development of a cataract induced by X-rays is a diminution of glutathione (GSH) level which begins before any clinical change can be detected (Pirie, van Heyningen & Boag, 1953). (This paper is to be considered as Part I of the series.) This decrease is accompanied by a decrease in the activity of glutathione reductase, but the activity of four other enzymes was not affected, even in the opaque lens. None of these four enzymes (lactic dehydrogenase, malic enzyme, cytochrome *c* reductase and *iso*citric dehydrogenase) is thought to contain functional sulphydryl (SH) groups.

Since protein sulphydryl is also diminished during the formation of cataract, though at a later stage than glutathione (Pirie *et al.* 1953), we have investigated the effect of X-irradiation of the eye of a rabbit, with consequent cataract formation, on three enzymes and one coenzyme, all known to depend on SH groups for their activity. The enzymes we have investigated are glyceraldehydephosphate dehydrogenase, which contains GSH as a prosthetic group (Krimsky & Racker, 1952), glyoxalase, whose substrate is a compound of glutathione and methylglyoxal (Racker, 1951), and an acetaldehyde oxidase, which is probably an SH enzyme. The activity of aldolase, a non-sulphydryl enzyme, was also determined.

It also seemed logical to try to assess the change in coenzyme A (CoA) after irradiation of the lens, since its sulphydryl group is essential for activity (Lynen, Reichert & Rueff, 1951). As the method of extraction of CoA from the lens by boiling is the same as that used to extract coenzymes containing nicotinic acid, we took the opportunity, whenever material was available, of estimating nicotinic acid as well as CoA. While it is possible that changes in the concentration of nicotinic acid would reflect changes in that of the active coenzymes diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN), we do not know what proportion of the nicotinic acid in lens is present in the form of these

coenzymes. Robinson, Levitas, Rosen & Perlzweig (1947) showed that, in rat liver, kidney and muscle, all the nicotinic acid was, in fact, in the form of DPN and TPN. A change in one or more of these coenzymes might be of particular importance, as they mediate so many steps in metabolism.

EXPERIMENTAL

Materials

Lens suspension and extract. These were prepared as before (Pirie *et al.* 1953).

Diphosphopyridine nucleotide (DPN). A crude preparation of nucleotides containing 30% DPN and 12% TPN was used (Lepage & Mueller, 1949).

Methods

Cataract was produced exactly as previously described, by irradiation of the right eye of a young rabbit, of known age, with X-rays (1400 r), leaving the left eye and lens barely affected. Change in enzymic activity and coenzyme content is expressed per lens rather than per g., as the weight of the irradiated lens changes, and we are interested in the change in the whole lens.

Reduced glutathione. GSH in the lens suspension was estimated by the method of Grunert & Phillips (1951). The total amount was measured in the right and the left lenses wherever possible, to supplement the slit-lamp and ophthalmoscopic assessment of cataract development. The GSH begins to diminish before any visible change is apparent, and continues to fall as the degree of opacity increases (Pirie *et al.* 1953). The concn. in the left lens (263–440 mg./100 g. wet wt.) was within the normal range. The percentage decrease in GSH after irradiation is given in Tables 1 and 3 in order that a comparison may be made between GSH loss and diminution of enzymes and coenzymes.

Glyceraldehyde phosphate dehydrogenase (GPD). This was estimated in Warburg manometers by measuring the rate of liberation of CO_2 from $NaHCO_3$ due to production of phosphoglyceric acid in the following reaction sequence:

Hexose diphosphate → glyceraldehyde phosphate (GP) + dihydroxyacetonephosphate (*aldolase*).

Dihydroxyacetonephosphate → GP (*triosephosphate isomerase*).

$2GP + 2DPN^+ \rightarrow 2$ phosphoglyceric acid $+ 2DPNH$ (*GPD*) (in presence of arsenate).

2 Pyruvate $+ 2DPNH \rightarrow 2$ lactate $+ 2DPN^+$ (*lactic dehydrogenase*).

Crystalline aldolase (prepared by the method of Cori, Slein & Cori (1948) and once recrystallized) and lactic dehydrogenase (kindly given by Dr D. M. Needham, F.R.S.) were added in excess so that the GPD in lens should be the limiting enzyme. No acid was formed in the absence of added lens. Arsenate was added, to enable the oxidation of GP to go to completion, and fluoride to inhibit phosphatases. The activity of the enzyme was always measured at two different concentrations, usually 0·3 and 0·6 ml. of 1:10 lens suspension in 3·0 ml. total vol., and the rate of acid production was proportional to the amount of lens. The enzymes were mixed with glutathione in the manometer flasks before adding the substrate, to ensure that any diminution of activity in the irradiated lens could not be due to the lowered concentration of GSH therein.

The contents of the manometer flasks were as follows: suitable dilutions of lens suspension, aldolase and lactic dehydrogenase, sodium arsenate (18 μmoles), DPN (approx. 0·15 μmole), sodium pyrophosphate (3 μmoles), glutathione (6·5 μmoles), $NaHCO_3$ (111 μmoles), KF (150 μmoles); total volume 2·6 ml. The side arm contained magnesium hexose diphosphate (12 μmoles) and sodium pyruvate (10 μmoles) in 0·4 ml. The gas phase was 95% N_2 + 5% CO_2 and the temperature 37°. The pH of the reaction mixture was calculated to be 7·8.

The activity of the enzyme is expressed as μl. CO_2 evolved/min. by the whole lens. The evolution was measured during 15–20 min.

Glyoxalase. This was measured in Warburg manometers by the method of Crook & Law (1952). Portions of 0·1 and 0·2 ml. of a 1:10 lens suspension were used and the rate of reaction was proportional to the amount of lens used. The activity of the enzyme is expressed as μl. CO_2 evolved/min. by the whole lens. The evolution was measured from 5 to 15 min. after starting the reaction.

Aldolase. This was measured at 37° by a modification of the method of Taylor, Green & Cori (1948). The activity of the enzyme is expressed as the μg. triose P formed/5 min. by the whole lens. The duration of the experiment was 15 min.

Acetaldehyde oxidase. This enzyme oxidizes acetaldehyde to acetic acid and requires DPN. The reduction of DPN was followed in a Beckman spectrophotometer in a 1 cm. cell at 340 mμ. The system consisted of lens extract (0·2–0·5 ml.), acetaldehyde (10 μmoles), DPN (0·14 μmole) and sodium pyrophosphate, pH 9 (60 μmoles); total volume 3·0 ml. The activity of the enzyme is expressed as 100 × the increase in the optical density ($\Delta \log I_0/I$) at 340 mμ./10 min. for the whole lens, readings being taken for 10 min. from 15 sec. after the addition of DPN, during which time the action was linear.

Acetic acid was estimated by the method of Bartley (1953).

Coenzyme A. The method of Kaplan & Lipmann (1948) was used, the sulphanilamide being estimated by the method of Bratton & Marshall (1939) and the CoA expressed as 'Lipmann units'. The lens was dissected from the eye, weighed and immediately dropped, whole, into a tared tube containing 1·5 ml. of 0·2M sodium acetate buffer, pH 5·7, standing in a boiling-water bath. After 3 min., the tube was removed, cooled and re-weighed. Any loss in weight due to evaporation was made up by addition of water, and the lens, which had usually partly disintegrated during the boiling, was rubbed up in the fluid with a glass rod. The suspension was centrifuged at 8000 *g*, and the CoA estimated in the supernatant fluid. The dilution of the lens in the sodium acetate was approximately 1:4 and 0·2–0·3 ml. were used for each test. Several dilutions of a standard CoA extract from liver were tested simultaneously each time the lens was tested. Owing to the small concentration of CoA in the lens extract, the mixtures were incubated at 37° for 3 hr., rather than for the 2 hr. recommended by Kaplan & Lipmann (1948).

Estimation of nicotinic acid. The lens was boiled as described for extraction of CoA and the supernatant fluid used. Nicotinic acid was determined by the method of Kodicek (1940), the final colour being read at 430 mμ. using the Beckman spectrophotometer.

RESULTS

Clinical course of formation of lens opacity

This has been described in the previous paper. The left lens was affected only very slightly, or not at all, and for simplicity we shall refer to it as normal (Pirie *et al.* 1953).

Changes in enzymes and coenzymes after irradiation

The results for the enzymes studied are given in Table 1. The rabbits are listed in decreasing order of severity of their cataracts, judged by slit-lamp and ophthalmoscopic appearance the day before death; the numbers are consecutive with those in our earlier paper. The order of cataract severity was decided by one of us before knowing the order of the changes of lens constituents. The lenses were opaque in nos. 21–24 inclusive. Nos. 25 and 26 showed major, but not complete, opacities and nos. 35, 36 and 37 showed only a trace of opacity in the lens.

Glyceraldehyde phosphate dehydrogenase and glyoxalase. The GPD activity of normal (left) lenses ranged from 63 to 183 μl. CO_2/min./lens and glyoxalase activity from 115 to 393 μl. CO_2/min./lens. The two enzymes were affected to roughly the same extent in any particular, irradiated lens. On two occasions (rabbits nos. 29 and 33), their activity was considerably diminished before gross changes in the

Table 1. *Comparison between right (irradiated) and left lenses. Changes in lens weight, concentration of reduced glutathione, and activity of glyceraldehyde phosphate dehydrogenase, glyoxalase, aldolase, and acetaldehyde oxidase*

(Rabbits are listed in order of decreasing severity of lens opacity. Methods of estimation of enzymic activity given in the Methods section.)

Rabbit no.		Lens weight (mg.)	Time after irradiation (weeks)	Decrease in irradiated lens (%)				
				GSH	Glyceraldehyde phosphate dehydrogenase	Glyoxalase	Aldolase	Acetaldehyde oxidase
21*	R	577	42	—	75	88	—	67
	L	499						
22*	R	631	43	—	100	100	—	—
	L	529						
23*	R	626	45	95	—	—	70	—
	L	548						
24*	R	611	26	82	28	22	—	61
	L	474						
25	R	494	17	87	—	—	—	42
	L	450						
26	R	448	25	70	15	16	—	45
	L	487						
27	R	389	32	61	—	41	29	—
	L	468						
28	R	447	32	48	—	23	0	—
	L	497						
29	R	463	22	—	52	38	—	18
	L	473						
30	R	457	22	37	0	6	—	12
	L	473						
31	R	475	21	38	9	6	0	40
	L	495						
32	R	470	20	49	—	9	0	—
	L	479						
33	R	450	16	49	40	46	0	—
	L	470						
34	R	398	18	40	—	—	—	23
	L	424						
35	R	441	17	22	0	11	—	30
	L	488						
36	R	420	15	32	—	—	—	24
	L	433						
37	R	436	17	9	—	—	—	10
	L	444						

* Lens opaque.

lens had occurred, but in rabbits nos. 24 and 26, where the cataract was well advanced, there was only slight loss of activity. Fig. 1 compares the activities of GPD in right and left lens of rabbit no. 29, and Fig. 2 compares the activity of glyoxalase in right and left lens of rabbit no. 33.

Aldolase. This enzyme was considerably affected only in the opaque lens (Table 1).

Acetaldehyde oxidase. This enzyme, which has not previously been described in the lens, is present in the lenses of both cattle and rabbits. It is similar to that described by Racker (1949) in ox liver. No attempt has been made to purify the enzyme, but some of its properties have been studied in lens extracts. Activity is assessed by measuring the increase in absorption at 340 mμ. in the presence of DPN. Under the conditions that we used, the enzyme reduces DPN not TPN; the pH optimum is near 9·0 and it has been established, using the method of Bartley (1953), that the product of the reaction is acetic acid.

We found that the enzyme was almost inactive in any buffer other than pyrophosphate, and at first considered that this showed that coenzyme A took part in the reaction (cf. Lipmann, Jones, Black & Flynn, 1952). However, no evidence for this could be obtained. Dialysis of the lens extract for 48 hr. against running tap water did not alter enzymic activity, although it would probably have removed the small amount of CoA present. Lynen *et al.* (1951) showed that incubation with 0·08 M iodoacetate for 2 hr. at room temperature inactivated CoA through combination of the iodoacetate with the SH group of the coenzyme. Such incubation before the testing of the DPN preparations used (one our own and one commercial), both of which were found to contain CoA, did not inhibit enzymic activity.

Pyrophosphate would seem to exert its activating effect through its chelating properties. The enzyme was almost inactive in phosphate, borate, glycine, veronal, and tris(hydroxymethyl)aminomethane buffers but was active in pyrophosphate, or in other buffers plus ethylenediaminetetraacetate. In the presence of small amounts of pyrophosphate, addition of Cu^{2+} inactivated the enzyme, but the addition of ethylenediaminetetraacetate prevented inactivation. These results are shown in Table 2. The pH optimum in ethylenediaminetetraacetate was near 9·0, as in pyrophosphate. The most active enzymic preparations were obtained by grinding and extracting the lens in the presence of ethylenediaminetetraacetate. We wish to thank Dr S. M. Altmann for bringing this effect of pyrophosphate on enzymes to our notice. Altmann & Crook (1953) found that chelating agents, among them pyrophosphate, markedly stimulated succinoxidase under certain conditions.

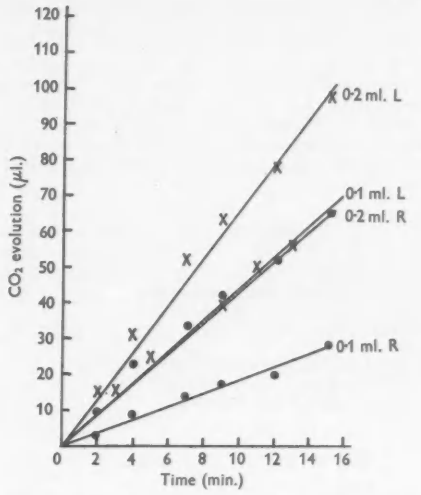

Fig. 1. Activity of glyceraldehyde phosphate dehydrogenase in right (irradiated) and left (normal) lens of rabbit no. 29.

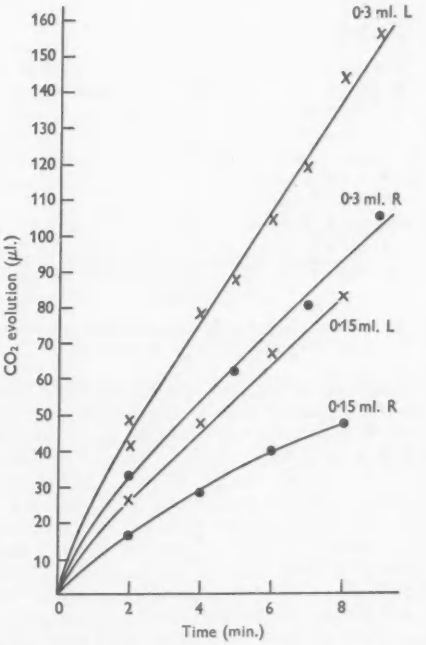

Fig. 2. Activity of glyoxalase in right (irradiated) and left (normal) lens of rabbit no. 33.

Table 2. *Activation of acetaldehyde oxidase by chelating agents and inactivation by copper*

(Activity was determined by measuring increase of absorption at 340 mμ. during 5 min. (ΔE). Each cell contained 0·4 ml. 1:4 cattle-lens extract, dialysed 48 hr.; acetaldehyde (10 μmoles), DPN (0·14 μmole), and other components as indicated, in a total vol. of 3·0 ml. All tests were at pH 9·0, the lens extract being adjusted before test.)

Addition	ΔE Expt. 1	ΔE Expt. 2
—	0·012	0·001
60 μmoles $Na_4P_2O_7$	0·084	0·118
16 μmoles ethylenediaminetetraacetate	0·104	0·134
60 μmoles $Na_4P_2O_7$ + 16 μmoles ethylenediaminetetraacetate	0·104	—
15 μmoles $Na_4P_2O_7$	0·077	—
15 μmoles $Na_4P_2O_7$ + 3 μmoles $CuSO_4$	0·014	—
15 μmoles $Na_4P_2O_7$ + 3 μmoles $CuSO_4$ + 16 μmoles ethylenediaminetetraacetate	0·109	—

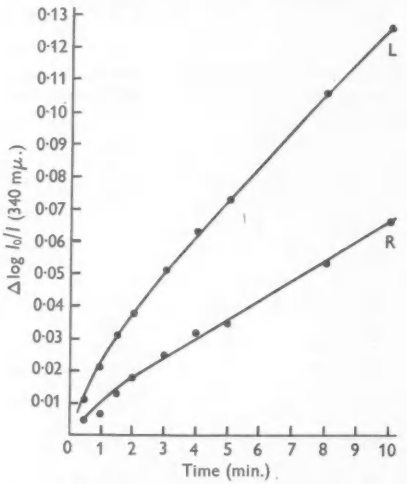

Fig. 3. Activity of acetaldehyde oxidase in right (irradiated) and left (normal) lens of rabbit no. 25.

Treatment of dialysed lens extract with iodoacetate for short periods at room temperature had no effect on activity, but after 2 hr. in the presence of 0·08 M iodoacetate activity dropped by about half. Immediate inactivation of the enzyme occurred if dialysed lens extract were treated with 3 mM iodoacetamide or with even lower concentrations (0·2–1·2 mM) of *o*-iodosobenzoate. Such inactivation took place in the presence of pyrophosphate or ethylenediaminetetraacetate. We take this as evidence that the activity of the enzyme depends on the presence of SH groups in its molecule.

Fig. 3 compares the activity of acetaldehyde oxidase in the right and left lenses of rabbit no. 25, the right lens showing a fall to about half the activity of the left. Table 1 shows that the activity of acetaldehyde oxidase is more regularly depressed at an early stage after irradiation than is that of glyceraldehydephosphate dehydrogenase, glyoxalase, or aldolase. The decrease in activity was the same whether pyrophosphate buffer, or phosphate buffer in the presence of ethylenediaminetetraacetate, was used. The decrease is, however, variable and is never complete. Rabbit no. 35, which showed a 30 % decrease in acetaldehyde oxidase activity in the right lens, was killed 4 months after irradiation and showed only traces of opacity in the lens, yet rabbit no. 30, killed 6 months after irradiation, had multiple peripheral opacities and yet the acetaldehyde oxidase activity was only diminished by 12 %. In rabbit no. 34, comparison was made between loss of aldehyde oxidase and loss of protein sulphydryl. It was found that protein SH had diminished by 11 % and the acetaldehyde oxidase by 23 %.

Coenzyme A after irradiation. The normal rabbit lens contains about 10 units CoA/g., about one-tenth of the concentration in liver (Table 3). The two lenses from a single normal animal differ by as much as 20 %, but this may be experimental error, which is large when such small amounts of coenzyme A are estimated. We found that the opaque lens contained little or no CoA, but that in the early stages of opacity there was little change. For example, rabbit no. 44, killed 23 weeks after irradiation, showed definite posterior opacities when examined with the ophthalmoscope, but the CoA content was a little higher in the irradiated than in the normal lens. The glutathione content of the irradiated lens had fallen by 39 %. It seems, therefore, that loss of CoA is not a primary change attendant on formation of opacity. The glutathione content of lenses used to estimate CoA and nicotinic acid was necessarily estimated in the boiled extract (see Methods). The values found were on the whole slightly higher than the values found in extracts prepared in the usual way.

Nicotinic acid after irradiation. Change in nicotinic acid followed much the same course as change in CoA (Table 3). No change was found when glutathione had already fallen, but by the time the lens was opaque there was a 77 % loss of nicotinic acid. Kodicek (1940) gave a value of 50 μg./g. of nicotinic acid in ox lens and found 100 μg./g. in a human cataract. He concluded that the nicotinic acid level was unaffected in cataract. Our results show that it is impossible to generalize, and that nicotinic acid does gradually fall in the rabbit lens during the formation of opacity after X-ray injury. Such a fall cannot, however, be considered as a cause of the opacity, as it takes place later than the fall in glutathione, and indeed after the opacities are clinically visible.

Table 3. *Change in coenzyme A and in nicotinic acid content in lens after irradiation*

(Rabbits listed in order of decreasing severity of lens opacity. Methods of estimation are described in Methods section.)

Rabbit no.		Lens weight (mg.)	Time after irradiation (weeks)	GSH decrease (%)	Coenzyme A		Nicotinic acid	
					Units per lens	Difference (%)	μg. per lens	Difference (%)
38*	R	518	44	—	Nil	—	—	—
	L	519			—		—	
39*	R	639	46	—	Nil	−100	—	—
	L	548			5·1		—	
40*	R	479	39	—	2·0	−64	25	−77
	L	510			5·6		113	
41*	R	467	28	—	1·2	−86	—	—
	L	518			9·0		—	
42	R	550	22	86	2·1	−53	29	−42
	L	512			4·5		49	
43	R	451	26	63	1·1	−70	23	−46
	L	516			3·7		43	
44	R	460	23	39	5·1	+8	28	−8
	L	482			4·7		30	
45	R	441	19	10	4·1	+13	33	−11
	L	441			3·6		37	
Normal	R	569	—	—	3·7	+9	30	−12
	L	560			3·4		35	
Normal	R	572	—	—	5·9	+22	—	—
	L	563			4·8		—	
Normal	R	443	—	—	4·5	—	37	−10
	L	446			4·6		41	

* Lens opaque.

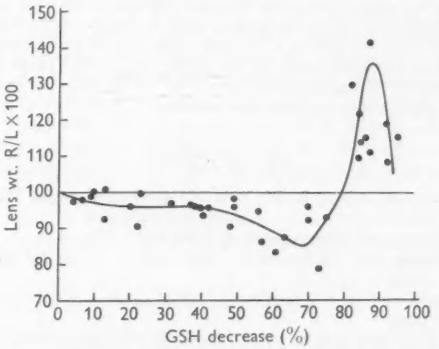

Fig. 4. Relation of change in weight of the irradiated lens to loss of glutathione.

Lens weight

In Fig. 4, the weight of the right lens is plotted against the loss in its GSH content, both values being expressed as percentage of that in the left lens. The figure includes points calculated from the data in Tables 1 and 3, others from data given in a previous paper (Pirie *et al.* 1953), and two further values from rabbits used in experiments outside the scope of this paper. Our previous suggestion, that in the early stages of formation of opacity the affected lens weighs less than the normal lens, is confirmed. There appears to be a rather sharp border line between this condition and a state in which the lens is intumescent and considerably heavier than the normal one. The change takes place when the GSH content has decreased by about 80 %. There is a suggestion that, as the cataract develops and the loss of GSH becomes even greater, the relative weight of the irradiated lens falls, but more observations are needed to confirm this.

DISCUSSION

The results reported here, together with those in our earlier paper (Pirie *et al.* 1953), support the suggestion of Bellows & Rosner (1937) and of Bellows & Schoch (1950) that loss of glutathione occurs early in cataract development. We have now studied altogether eight different enzymes in lens after irradiation, and the results, in general, show that, of these particular enzymes, those activities which depend on sulphydryl groups are affected before those which are not so dependent. We found that cytochrome *c* reductase, lactic dehydrogenase, *iso*citric dehydrogenase, and malic enzyme, are normally active in the opaque lens (Pirie *et al.* 1953), and aldolase only loses activity in the very late

stages. These enzymes are not dependent on sulphydryl groups. On the other hand, glyceraldehyde phosphate dehydrogenase, glyoxalase, and acetaldehyde oxidase, which progressively lose activity during the formation of opacity, need sulphydryl groups. Change in the enzymic activity occurs later than change in glutathione concentration, which suggests that the concentration of enzyme sulphydryl groups is maintained to some extent at the expense of glutathione.

The tests of activity of those enzymes needing glutathione, either as a coenzyme or as a stabilizer, have been made in the presence of an added excess of glutathione. Fall in enzymic activity is therefore not simply due to a fall in the glutathione content, although it may be a consequence of this. If glutathione acts as a stabilizer of protein SH, then the enzymes may become irreversibly inactivated when the glutathione in the lens falls below a certain level. It is possible that this is the reason why enzymes dependent on sulphydryl groups appear to be more susceptible to damage.

Glutathione reductase appears to be an exception, in that loss of activity can be detected very early in cataract formation, and it is doubtful whether it is an enzyme activated by sulphydryl groups (van Heyningen & Pirie, 1953). It is possible that the loss of glutathione in the irradiated lens is due both to fall in the activity of glutathione reductase and to a decrease in the rate of its synthesis, as described by Kinsey & Merriam (1950) in naphthalene-induced cataract.

With the small amount of tissue available, it is difficult to estimate the coenzyme content with accuracy, but it appears as if coenzyme A, and also nicotinic acid, diminish progressively in the lens as the opacity develops, but no striking change occurs at an early stage.

Apart from changes in certain enzymes and coenzymes, our results show that a general change must take place before clinical opacities are advanced. The irradiated lens may be 5–10 % lighter than the lens in the normal fellow-eye when only very slight changes can be seen with the slit-lamp or ophthalmoscope. Salit (1939) and Bloch & Salit (1946) have shown that, in development of nuclear sclerosis or of cataract in the human lens, there is a loss of lens protein. Salit, Swan & Paul (1942) showed that in rats fed with galactose, the wet weight of the lens fell progressively during the development of cataract and, at the same time, the dry weight fell even more sharply, until a completely opaque lens had only 31 % of the dry weight of a normal lens. Merriam & Kinsey (1950) calculated from studies with radioactive glycine that the turnover of protein in the lens of the rabbit is 2·5–5 %/day. It is possible that the loss in weight of the lens after irradiation may be explicable as a failure of protein synthesis.

We must record that all the changes we have described occur some time after irradiation. They provide no direct evidence on the initial physical and chemical changes in the lens caused by the radiation, but they are of interest in the general study of cataract production.

SUMMARY

1. Glyceraldehyde phosphate dehydrogenase, glyoxalase, acetaldehyde oxidase, and aldolase, are present in the rabbit lens.

2. The activities of glyceraldehyde phosphate dehydrogenase, glyoxalase, and acetaldehyde oxidase, fall during development of opacity in the lens after X-irradiation.

3. The activity of aldolase is unchanged except in the opaque lens, when it is diminished.

4. The content of coenzyme A and of the nicotinic acid falls gradually during the development of opacity.

5. The weight of the irradiated lens is less than that of the fellow-eye during the early stage of opacity formation.

6. These results are discussed in relation to the known changes in glutathione and in protein sulphydryl groups in cataract.

We wish to thank the Medical Research Council for an expenses grant, the National Council to Combat Blindness, Inc., for the gift of a Beckman spectrophotometer, and Miss Joyce Blackwell for her skilful care of the rabbits. Finally we record our gratitude to Dr H. Gray for the unfailing stimulus of his co-operation.

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Metabolism of Thiamine Phosphates in Washed Suspensions of Kidney Particles

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Whilst it is certain that the oxidation of pyruvate and α-oxoglutarate in animal tissues requires, among other cofactors, cocarboxylase (Lohmann & Schuster, 1937; Barron, Goldinger, Lipton & Lyman, 1941; Stumpf, Zarudnaya & Green, 1947), the precise role of this coenzyme is still obscure. As the oxidative decarboxylation of α-ketonic acids is accompanied by the synthesis of pyrophosphate bonds of ATP, it seemed possible that cocarboxylase might act as a phosphate carrier (Kiessling & Lindhal, 1952). This hypothesis became capable of experimental testing with the availability of isotopic phosphorus and of methods capable of separating small quantities of organic phosphates by paper chromatography (Hanes & Isherwood, 1949; Eggleston & Hems, 1952; Bartley, 1953*b*; Krebs & Hems, 1953). Experiments are reported in this paper in which the rate of incorporation of $^{32}PO_4$ into thiamine phosphates was measured and compared with the rate of oxidation of α-ketonic acids.

EXPERIMENTAL

Special chemicals. Thiamine triphosphate, thiamine diphosphate, oxythiamine diphosphate and thiamine were commercial samples (Roche Products Ltd.) of more than 95% purity. Thiamine monophosphate was prepared by the method of Karrer & Viscontini (1946) and oxythiamine by the method of Rydon (1951). Adenosine triphosphate (ATP) preparations contained about 80% ATP and 10% adenosine diphosphate (ADP). α-Oxoglutarate was prepared by the method of Friedman & Kosower (1946) and was not less than 98% pure, as estimated by the method of Krebs (1950). Pure sodium pyruvate was prepared according to Robertson (1942). All other reagents were of analytical grade.

Chemical estimations. Keto-acids were estimated by the 'specific' extraction method of Friedemann & Haugen (1943), succinate manometrically according to Krebs (1937), and free thiamine directly in the trichloroacetic acid (TCA) filtrates by the method of Melnick & Field (1938), the optical density of the red colour extracted into xylene being measured spectrophotometrically at 520 mμ. Measurements of radioactivity were made on a sample of the diluted, wet-ashed material or on the *iso*butanol extract obtained by the phosphate estimation. An M/6 liquid counter (20th Century Electronics) was used.

Preparation and incubation of the particulate suspensions. Particulate suspensions of sheep-kidney cortex, consisting mainly of mitochondria suspended in 0·9% (w/v) KCl, were prepared as described by Bartley (1953*a*). In all cases the 'R_3 residue' was used. Samples (2 ml.) of the tissue suspension (60–100 mg. dry wt.) were added to 2 ml. of medium in the main compartment of conical Warburg vessels which included ATP, $MgCl_2$ (both at 0·001 M final concn.) and phosphate buffer, pH 7·4 (final concn. usually 0·005 M). The centre wells contained NaOH and filter paper, with O_2 in the gas space. The O_2 uptake was measured at 40° and the incubation was stopped at varying times by transfer of the vessel to ice-water and addition of 0·5 ml. 30% (w/v) TCA to each vessel. The precipitated protein was removed by centrifugation and the TCA extract was stored at −18° until it could be analysed.

Paper-chromatographic separation, identification and estimation of thiamine phosphates. The method of Viscontini, Bonetti, Ebnöther & Karrer (1951) proved unsuitable, as it failed to separate thiamine mono- and di-phosphates, and a new method was therefore elaborated. Whatman no. 1 filter paper washed with ethylenediaminetetraacetic acid (Eggleston & Hems, 1952) was used throughout. Of several solvent systems tested, namely benzyl alcohol/formic acid, cellosolve/water, dioxan, *tert.*-pentyl alcohol/formic acid, *iso*propyl ether/formic acid and *p*-toluenesulphonic acid/*tert.*-pentanol/water (Hanes & Isherwood, 1949), only the last was satisfactory. Solutions were delivered from an Agla syringe (Burroughs Wellcome Ltd.) either as spots or as bands along a line 8 cm. from the end of a rectangular filter paper, 18 × 45 cm. After drying in a current of cold air, the papers were irrigated with the solvent by descending

chromatography in an all-glass apparatus, usually for 17 hr. Three methods were used to render thiamine derivatives visible on the paper. (1) Examination in ultraviolet light (260 mμ.). This is unspecific and, moreover, the absorption of small amounts of thiamine derivatives was obscured by the absorption of the *p*-toluenesulphonic acid in the solvent system. (2) Spraying with the molybdate reagent of Hanes & Isherwood (1949) which is applicable to phosphorylated derivatives. (3) Using the colour reaction with potassium bismuth iodide (Naimans, 1937) which is relatively specific for thiamine compounds. The spray reagent, which keeps indefinitely, is prepared by boiling about 5 g. of potassium bismuth iodide (B.D.H. reagent grade) in 100 ml. distilled water containing 0·5 ml. conc. HCl and filtering the orange solution when cold. When the dried chromatograms are sprayed with the reagent, thiamine derivatives appear as red-purple to orange spots, the tint varying with the compound. The chromatograms are viewed against a white light while still wet, as the red colour of the thiamine spots tends to be obscured by the orange background after drying. To obtain maximum colour development it is an advantage to re-spray the paper after drying. The colour of the background of the dried chromatogram may be removed by laying the paper in a photographic developing dish containing diethyl ether; a little water is added after immersion of the paper and the dish is gently rocked. The potassium bismuth iodide is, under these conditions, extracted by the ether layer and leaves behind the red or orange thiamine

Table 1. *Values of R_F, $R_{thiamine}$, and colour reactions with potassium bismuth iodide of some thiamine derivatives and of some other nitrogen-containing substances*

(5 μl. of approximately 0·05 M solutions of the substances were chromatographed as described in the text. The colours recorded were those observed by viewing in a white light. Substances were commercial preparations and in some cases contained other substances; the major constituent is indicated by an asterisk and the components are labelled in sequence *a*, *b*, etc. As the R_F values are somewhat variable, the $R_{thiamine}$ (distance travelled by substance/distance travelled by thiamine) values are also given.)

Substance	R_F	$R_{thiamine}$	Appearance under u.v. light	Colour when sprayed	Colour after washing, as described in text
Thiamine triphosphate	0·08	0·11	Absorbs	Orange	Orange
Thiamine diphosphate (cocarboxylase)	0·18	0·25	Absorbs	Orange-pink	Orange-pink
Oxythiamine diphosphate	0·11	0·16	Absorbs	Orange	Orange
Thiamine monophosphate	0·42	0·59	Absorbs	Pink-orange	Pink-orange
Thiamine	0·71	1·00	Absorbs	Pink	Pink
Oxythiamine	0·41	0·58	Absorbs	Pink-purple	Pink-purple
Chlorothiamine	0·82	1·15	Absorbs	Purple-pink	Purple-pink
5-(β-Hydroxyethyl)-4-methylthiazole	0·64	0·90	Absorbs	Faint purple-pink	None
5-Ethoxy-4-methylthiazole	0·64	0·90	Absorbs	Transient pink	None
2:4-Dimethyl-5-(β-hydroxyethyl)-thiazole. Two components *a**	0·65	0·91	Absorbs	Pale-pink	Almost invisible, pale-pink
2:4-Dimethyl-5-(β-hydroxyethyl)-thiazole. Two components *b*	0·87	1·22	Absorbs	Pale-pink	Almost invisible, pale-pink
5-(β-Acetoxyethyl)-4-methylthiazole	0·72	1·01	Absorbs	Very faint pink	None
4-Amino-5-cyano-2-methylpyrimidine	0·45	0·63	Faintly absorbs	None	None
Pyrimidine	0·53	0·75	Absorbs	None	None
4-Amino-2-methyl-5-thioformylamino-methylpyrimidine	0·75	1·05	Absorbs	Pink	Pink
Caffeine	0·77	1·08	Absorbs	None	None
Morphine sulphate. Two components *a**	0·68	0·96	Absorbs	Red	None
Morphine sulphate. Two components *b*	0·85	1·20	Absorbs	Red	None
Quinine hydrochloride	0·80	1·12	Intense-white fluorescence	Pink-orange	Pink-orange
Strychnine hydrochloride	0·82	1·15	Absorbs	Orange	Orange
Brucine hydrochloride	0·68	0·95	Absorbs	Orange	Orange
Cinchocaine hydrochloride	0·85	1·20	Intense-white fluorescence	Red	Red
Mepyramine	0·78	1·09	Intense-white fluorescence	Red	Red
Quinoline hydrochloride. Two components *a**	0·68	0·96	Intense-white fluorescence	Pink	Pink
Quinoline hydrochloride. Two components *b*	0·84	1·18	Intense-white fluorescence	Pink	Pink
Nicotine	0·55	0·77	Absorbs	Red-purple	Faint red
Choline chloride	0·45	0·63	Not visible	Purple	Purple-brown
Acetyl choline chloride	0·53	0·75	Not visible	Purple-blue	None
Benzoyl choline chloride. Three components *a*	0·4	0·57	Not visible	Purple	Purple-brown
Benzoyl choline chloride. Three components *b**	0·76	1·07	Fluorescence	Pink	Orange
Benzoyl choline chloride. Three components *c*	0·94	1·32	Not visible	Pink	Pink
Betaine hydrochloride	0·38	0·75	Slightly absorbs	Pale-blue	None
Agmatine sulphate	0·18	0·24	Very faintly absorbs	None	None

bismuth iodide complex. No extraction into the ether occurs until the water is added. The papers may be dried and kept as records. The method is not suitable if analysis of the phosphate on the chromatogram is required as some phosphate compounds disappear during the washing procedure. As little as 0·1 μg. of thiamine can be detected by this method. The substances tested with the potassium bismuth iodide reagent are listed in Table 1. Several compounds containing basic nitrogen react and the colour obtained appears to vary with the basicity of the nitrogen, becoming bluer the more basic the nitrogen, the bluest being obtained with the quaternary ammonium derivatives, such as choline. Esterification of choline with benzoic acid (benzoyl choline), which reduces the basicity of the nitrogen, changes the colour to red. In the experiments described in this paper, thiamine compounds were the only substances reacting with potassium bismuth iodide.

Of the three methods for locating the thiamine derivatives, the ultraviolet absorption has the advantage that the material remains unchanged and may be eluted from the paper for chemical estimations. The papers sprayed with molybdate may be used for phosphorus determinations as described by Bartley (1953*b*) and the papers treated with the potassium bismuth iodide reagent may subsequently be sprayed with molybdate and used for the determination of phosphorus, as potassium bismuth iodide does not interfere. The phosphate-containing compounds separated on paper were analysed as described by Bartley (1953*b*).

RESULTS

Effect of thiamine derivatives on respiration. The addition of thiamine, thiamine monophosphate, cocarboxylase, oxythiamine, oxythiamine diphosphate (all 0·001 M) or of thiamine, thiamine monophosphate or cocarboxylase (0·005 M) did not markedly alter the respiration or the ratio of oxygen uptake/pyruvate consumed ('O_2/pyruvate ratio') of a sheep-kidney suspension oxidizing pyruvate, but the addition of 0·005 M thiamine triphosphate inhibited respiration by 40% without altering the above ratio.

Metabolism of cocarboxylase during the oxidation of pyruvate. When cocarboxylase was incubated with sheep-kidney suspensions, cocarboxylase disappeared and other phosphate-containing compounds were formed. A quantitative experiment is shown in Table 2. Three new phosphorus-containing compounds were present at the end of the incubation in addition to the ATP, cocarboxylase and inorganic orthophosphate added to the incubation mixture. The six phosphorus compounds present are numbered in Table 2 on the basis of the R_F values. In another experiment in which the sodium bicarbonate concentration was lowered to 0·01 M, spots 1 and 4 were not formed and the amount of cocarboxylase broken down was much greater in the absence of pyruvate than in its presence (see Table 3).

Spot 3 behaved like a pure sample of thiamine monophosphate in the following tests: (1) the R_F values in the *p*-toluenesulphonic acid/*tert.*-pentanol/water mixture were the same, i.e. approximately 0·42 (R_F of cocarboxylase, 0·18; thiamine, 0·7). (2) The molybdate spray of Hanes & Isherwood (1949) gave the same pale-blue colour which slowly increased with time, reaching a maximum in about

Table 2. *Formation of phosphorus compounds on addition of cocarboxylase to a sheep-kidney mitochondrial suspension*

(The incubation mixture contained 0·0125 M pyruvate, 0·0125 M-$NaHCO_3$, 0·0075 M phosphate buffer, pH 7·4, 0·001 M-$MgCl_2$, 0·001 M-ATP, and 2 ml. of a suspension of kidney particles in 0·9% (w/v) KCl; final volume 4 ml.; incubation 60 min. at 40° in O_2.)

Spot no.	R_F	Compound	Phosphorus present: Before incubation (μg.)	Phosphorus present: After incubation (μg.)
1	0·68	Phosphopyruvate	0	134
2	0·54	Inorganic orthophosphate	930	1110
3	0·42	Thiamine monophosphate	0	171
4	0·23	Unknown	0	53
5	0·18	Cocarboxylase	496	175
6	0·09	ATP + ADP	387	59
		Total	1813	1702

Table 3. *Effect of pyruvate on decomposition of cocarboxylase in a sheep-kidney mitochondrial suspension*

(0·01 M-$NaHCO_3$; pyruvate when present, 0·0125 M; phosphate buffer pH 7·4, 0·005 M; other additions as in Table 2; incubation 40 min. at 40°.)

Spot no.	R_F	Compound	Phosphorus present: Before incubation (μg.)	Phosphorus present: After incubation with pyruvate (μg.)	Phosphorus present: After incubation without pyruvate (μg.)
2	0·54	Inorganic orthophosphate	632	659	1248
3	0·42	Thiamine monophosphate	0	Just detectable	65
5	0·18	Cocarboxylase	730	616	387
6	0·09	ATP + ADP	615	316	110
		Total*	1977	1601	1810

* Small amounts of other phosphates, for example AMP, were formed, but these were not estimated.

48 hr. Exposure to ultraviolet light or prolonged heating after spraying shortened the time for maximum colour formation to about 1 hr. (3) Spraying with a solution of potassium bismuth iodide gave a pink-orange colour, not extractable by ether.

By elution with water from several chromatograms, 5 ml. of a solution were obtained, which contained no free orthophosphate but about 100 μg. total phosphorus. This solution and a solution of pure thiamine monophosphate showed the following properties in common: (*a*) No phosphate was liberated on heating for 1 hr. in 2N-H_2SO_4 at 100°. (*b*) No xylene-soluble pigment was formed on treatment with diazotized *p*-aminoacetophenone. (*c*) All the phosphate was liberated on incubation with alkaline phosphatase in 2 hr. at 40° in bicarbonate buffer, pH 8·0. (*d*) Alkaline phosphatase liberated free thiamine which was identified and estimated by coupling with diazotized *p*-aminoacetophenone to give a red, water-insoluble pigment which could be extracted into xylene (Melnick & Field, 1938). (*e*) The amount of thiamine liberated by alkaline phosphatase was about 75% of the amount expected on the basis of the amounts of phosphate released; however, considering that thiamine is unstable at pH 8·0, a deficit is expected.

It may thus be concluded that spot 3 was thiamine monophosphate, and it is referred to as such in the remainder of this paper.

Spot 1, as shown in the following paper, was phosphopyruvate. Spot 4 was unidentified.

In order to measure the changes in the phosphorylated compounds, cocarboxylase was incubated for varying periods (5–40 min.) in a series of Warburg vessels. Each vessel contained 20 μmoles of sodium bicarbonate (0·005M) and 32·4 μmoles of sodium pyruvate (0·0081M). ^{32}P-Labelled orthophosphate was added in order to examine whether the various phosphates incorporated inorganic phosphate. Data on the oxygen uptake, substrate changes, distribution of phosphorus and of radioactivity are shown in Tables 4 and 5. According to

Table 4. *Oxygen uptake, pyruvate consumption and changes in phosphorus-containing substances occurring on incubation of cocarboxylase with sheep-kidney mitochondria*

(Each flask contained initially 20 μmoles of $NaHCO_3$ and 32·4 μmoles of sodium pyruvate; analytical data refer to the total contents of the flask (4·0 ml.).)

Time of incubation (min.)	Vessel no.	O_2 uptake (μmoles)	Pyruvate consumption (μmoles)	Ortho-phosphate present (μmoles)	Cocarboxylase phosphorus present (μmoles)	ATP + ADP phosphorus present (μmoles)	Thiamine monophosphate (spot 3) formed (μmoles)
0	1	0	0	21·2	33·2	8·3	0
5	2	4·15*	3·6	26·2	30·6	3·0	2·0
10	3	8·30*	8·9	24·7	30·3	4·6	2·3
15	4	12·65	14·0	26·8	26·4	4·3	4·2
20	5	15·95	18·1	28·6	22·3	5·8	5·2
25	6	23·60	22·5	28·2	23·6	4·2	5·8
30	7	27·80	25·7	27·2	19·9	5·0	10·0
35	8	26·40	26·6	30·4	20·5	3·2	7·9†
40	9	36·20	27·4	35·3	17·8	3·8	5·0

* O_2 uptake obtained by extrapolation from the average O_2 uptake of vessels 5–9.
† Free thiamine detected on the paper chromatogram at this time.

Table 5. *Incorporation of ^{32}P-labelled orthophosphate into other phosphorus-containing compounds on incubation of cocarboxylase with sheep-kidney mitochondria*

(Same experiment as Table 4)

	Specific radioactivity (counts/min./μg. P) in				
Time of incubation (min.)	Orthophosphate	Cocarboxylase	Adenosine polyphosphates	Labile P of adenosine polyphosphates calculated as all ADP	Thiamine monophosphate
0	1685	0	0	0	0
5	1430	11	411	822	113
10	1340	8	479	958	128
15	1330	20	537	1074	53
20	1290	21	474	948	133
25	1260	35	566	1132	82
30	1170	27	632	1264	61
35	1120	46	432	864	108
40	1080	27	472	944	77

Table 4, the quantity of inorganic orthophosphate progressively increased, whilst that of cocarboxylase progressively decreased. The quantities of ATP+ADP phosphorus fell rapidly within the first 5 min. and then remained fairly constant. Thiamine-monophosphate phosphorus increased to a maximum at 30 min. and then decreased with formation of free thiamine. Table 5 shows that the incorporation of ^{32}P into cocarboxylase was almost negligible. In contrast, incorporation into adenosine polyphosphates was very rapid, but slower than expected (see Krebs, Ruffo, Johnson, Eggleston & Hems, 1953; Bartley, 1953*b*), reaching full equilibrium only after 30 min., instead of 3–4 min. Compared with cocarboxylase, thiamine monophosphate incorporated ^{32}P fairly rapidly, but no equilibrium was reached for reasons that will be discussed later. The correlation of the quantitative aspects (Table 6) shows no consistent relationship between the amount of cocarboxylase broken down and of pyruvate oxidized. These results indicate that added cocarboxylase is not a phosphate carrier in the oxidation of pyruvate.

Metabolism of thiamine and thiamine monophosphate in the presence of pyruvate or fumarate. No formation of thiamine monophosphate could be demonstrated when kidney particles were incubated for up to 40 min. with a range of thiamine concentrations up to 0·005M in the presence of pyruvate or fumarate (0·0125M) and sodium bicarbonate (0·005M). When thiamine was replaced by thiamine monophosphate, no cocarboxylase could be detected by paper chromatography, and about 50% of the added thiamine monophosphate was hydrolysed to free thiamine after 30 min. Spot 1 again appeared. These results suggest that a thiamine phosphorylating enzyme of the type described by Nguyen Van Thoai & Chevillard (1949) in liver and by Steyn-Parvé (1952) in yeast was absent from the suspension, as expected on the basis of the findings of Nielsen & Leuthardt (1950) who showed that the enzymes which phosphorylate thiamine are present in the soluble fraction of rat liver and absent from the mitochondria.

Table 6. *Relationship between cocarboxylase disappearance and pyruvate utilization during the oxidation of pyruvate by sheep-kidney mitochondria*

(The values given are calculated from the data in Table 4.)

Time (min.)	Cocarboxylase removed (μmoles)	Pyruvate removed (μmoles)	Ratio: pyruvate removed / cocarboxylase removed
5	2·6	3·6	1·4
10	2·9	8·9	3·1
15	6·8	14·0	2·1
20	10·9	18·1	1·7
25	9·6	22·5	2·3
30	13·3	25·7	1·9
35	12·7	26·6	2·1
40	15·4	27·4	1·8

Metabolism of cocarboxylase in the presence of α-oxoglutarate and succinate. The sheep-kidney suspensions used in the experiments so far discussed contained up to 0·0125M bicarbonate, this being necessary for the complete oxidation of pyruvate. During incubation the pH of the medium increased from 7·4 to 8·0. To ensure that the results obtained were not due to high pH, pyruvate and bicarbonate were replaced by α-oxoglutarate or succinate. The respiration, substrate consumption and changes in the distribution of phosphorus are given in Table 7 and the specific activities of the phosphorus-containing compounds are given in Table 8. Again, the incorporation of radioactivity into cocarboxylase was very slight and the specific radioactivity of the cocarboxylase was rather smaller with α-oxoglutarate than with succinate, although the oxidation of α-oxoglutarate is known to require cocarboxylase (Barron *et al.* 1941; Stumpf *et al.* 1947). The formation of thiamine monophosphate was not affected by the presence of substrate, but the incorporation of radioactivity into thiamine monophosphate was accelerated by succinate and α-oxoglutarate and was 3–4 times faster than in the presence of pyruvate. With succinate, the incorporation was progressive, with α-oxoglutarate it reached a maximum within 10 min. The breakdown of cocarboxylase was slow when α-oxoglutarate was present, but when the α-oxoglutarate had virtually disappeared (after 20 min.) the amount of cocarboxylase that disappeared was about the same as that which disappeared in the presence of succinate or in the absence of substrate. This parallels the earlier finding (Table 4) that pyruvate diminished the rate of breakdown of cocarboxylase.

Foa, Weinstein, Smith & Greenberg (1952) have shown a similar inhibition of the breakdown of endogenous thiamine phosphates by the addition of insulin, but as no distinction was made between cocarboxylase and thiamine monophosphate it is uncertain whether the dephosphorylation of cocarboxylase or of thiamine monophosphate was inhibited.

In the absence of bicarbonate, thiamine monophosphate was stable. The decomposition in the presence of bicarbonate is probably due to the higher pH. However, after incubation of kidney particles with thiamine monophosphate (up to 0·0025M) at pH 7·4 with α-oxoglutarate or succinate as substrate, and in the absence of bicarbonate, for periods up to 1 hr., no cocarboxylase could be detected by paper chromatography. When radioactive phosphate was added to the incubation medium there was no radioactivity associated with the area of the paper chromatogram where cocarboxylase would be expected.

Table 7. *Metabolism of cocarboxylase in sheep-kidney mitochondria*

(In Expt. 1 the substrate was 57·6 μmoles of α-oxoglutarate; cocarboxylase added approx. 0·002 M. In Expt. 2 substrate was 65·6 μmoles of succinate; cocarboxylase added approx. 0·00175 M. In Expt. 3 no substrate was added; cocarboxylase added approx. 0·0015 M. Other additions as in Table 2. The analytical data refer to the total content of each Warburg vessel (4 ml.).)

Expt. no.	Substrate added	Time of incubation (min.)	O_2 uptake (μmoles)	Quantity of added substrate removed (μmoles)	Ortho-phosphate found (μmoles)	Cocarboxylase phosphorus found (μmoles)	ATP + ADP phosphorus found (μmoles)	Thiamine monophosphate phosphorus found (μmoles)
1	α-Oxo-glutarate	0	0·0	0·0	18·2	16·9	8·30	0·00
		10	24·8	40·2	17·4	16·0	5·00	1·61
		20	51·1	55·4	21·0	13·1	5·64	3·02
		30	84·3	56·1	16·6	7·7	5·58	2·15
2	Succinate	0	0·0	0·0	19·2	13·5	7·8	0·00
		10	25·6	45·8	19·2	8·9	2·8	2·03
		20	58·3	55·4	17·4	7·4	4·4	3·35
		30	80·9	54·7	14·9	6·5	4·6	4·14
3	None	0	0·0	0·0	16·0	11·6	7·3	0·00
		10	2·1	0·0	22·2	7·0	0·83	2·11
		20	2·8	0·0	25·6	4·8	0·00	2·92
		30	3·3	0·0	26·1	3·3	0·00	4·39

Table 8. *Incorporation of ^{32}P-labelled orthophosphate into other phosphorus-containing compounds on incubation of cocarboxylase with sheep-kidney mitochondria*

(Same experiments as described in Table 7.)

			Specific radioactivity (counts/min./μg. P) in				
Expt. no.	Substrate added	Time of incubation (min.)	Orthophosphate	Cocarboxylase	Adenosine polyphosphates	Labile P of adenosine polyphosphates calculated as all ADP	Thiamine monophosphate
1	α-Oxoglutarate	0	2088	0	0	0	0
		10	1630	5	683	1366	442
		20	1265	5	525	1050	305
		30	1235	35	662	1324	452
2	Succinate	0	1655	0	0	0	0
		10	1211	12	389	778	95
		20	1150	20	428	956	314
		30	1295	77	666	1332	633
3	None	0	1546	0	0	0	0
		10	1022	10	99	198	65
		20	894	12	0	0	58
		30	836	40	0	0	—

Table 9. *Incorporation of ^{32}P-labelled orthophosphate into thiamine monophosphate*

(Each vessel contained 6·9 μmoles of thiamine monophosphate, 55·7 μmoles of α-oxoglutarate and about 70 mg. dry wt. of kidney particles; other additions as in Table 2.)

Time of incubation (min.)	Ratio: $\frac{A^*}{B} \times 100$.
0	0·0
5	5·4
10	7·5
15	12·0
20	13·3
30	16·5
60	35·0

* $\frac{A}{B} = \frac{\text{specific activity of thiamine monophosphate}}{\text{specific activity of orthophosphate}}$.

It was thought that cocarboxylase might not become radioactive because it might not penetrate into the mitochondria. However, this assumption was not borne out by direct measurement of the distribution of cocarboxylase between mitochondria and medium by the methods described by Bartley & Davies (1954). This gave a ratio of the cocarboxylase in the mitochondria/the cocarboxylase in the medium of 0·8 after 10 min. at 20°.

Turnover of thiamine monophosphate. The rate of incorporation of ^{32}P-labelled orthophosphate into thiamine monophosphate was measured at pH 7·4 with α-oxoglutarate as the substrate. Under these conditions the concentration of thiamine monophosphate was found to remain constant. The rate of incorporation of radioactivity into thiamine monophosphate (Table 9) shows that ^{32}P is incorporated

at a slow but steady rate of about 0·6 % (0·053 μ-moles)/min.

It has already been shown that the kidney suspensions do not phosphorylate thiamine when pyruvate plus bicarbonate was the substrate, and that added thiamine monophosphate is dephosphorylated. Phosphorylation of thiamine was also tested under conditions where thiamine monophosphate was stable (pH 7·4 with α-oxoglutarate as substrate) but no evidence of phosphorylation was found. The radioactivity of the thiamine monophosphate area found after 1 hr. indicated less than 4 μg. thiamine monophosphate compared with the exchange rate of added thiamine monophosphate of about 80 μg./hr. Thus, thiamine is not appreciably phosphorylated in the mitochondria under the conditions tested.

DISCUSSION

Incorporation of ^{32}P-labelled orthophosphate into cocarboxylase. Although incubation of respiring mitochondria with cocarboxylase and ^{32}P-labelled orthophosphate leads to the appearance of the labelled phosphorus in cocarboxylase, the incorporation is very slight and bears no constant relationship to the extent of substrate oxidation (Table 10). It is therefore very unlikely that cocarboxylase acts as a phosphate carrier. Kiessling & Lindahl (1952) have already claimed to have demonstrated the incorporation of ^{32}P into cocarboxylase in mitochondrial suspensions containing $KH_2{}^{32}PO_4$, but it is uncertain whether the isolated cocarboxylase contained thiamine monophosphate, a point raised by the results reported in this paper. Kiessling & Lindahl also report a 'fair' proportionality between the rate of incorporation and of oxygen consumption when pyruvate was the substrate. This does not hold when different ketonic acids are compared (Table 10).

Route of formation of thiamine monophosphate. The fact that the specific activity of thiamine monophosphate always remained below that of the labile P of ATP and inorganic P could be accounted for by the assumption that thiamine monophosphate can be formed from cocarboxylase by more than one route. If it were formed only by dephosphorylation of the cocarboxylase to thiamine and rephosphorylation to the monophosphate, it would have at least the same specific activity as the ATP or inorganic P at the same time (whichever was the lower). Thiamine monophosphate formed directly by the hydrolysis of one phosphate group of cocarboxylase cannot be radioactive. The occurrence of these two reactions could account for the observed specific activities of the thiamine monophosphate, but as thiamine was not phosphorylated by the preparations used, thiamine monophosphate cannot be formed by a route involving free thiamine, and the above explanation therefore cannot be correct. However, the facts are in agreement with the assumption that thiamine is rephosphorylated in some combined form. Reed & DeBusk (1952) have shown that 'lipothiamide pyrophosphate' is the active form of cocarboxylase for some strains of *Escherichia coli*. If this holds also for animal tissues, then its formation must readily occur because cocarboxylase can fully re-activate the cocarboxy-

Table 10. *Relations between the amounts of phosphorus of cocarboxylase exchanged and the amounts of substrate oxidized*

(Phosphorus exchange calculated from the data given in Tables 5 and 8.)

Substrate	Incubation time (min.)	Cocarboxylase P exchanged (μmoles)	Substrate removed (μmoles)	Ratio: substrate removed/ cocarboxylase P exchanged
Pyruvate	5	0·254	3·6	14·5
	10	0·198	8·9	44·9
	15	0·500	14·0	28·0
	20	0·540	18·1	33·5
	25	0·923	22·5	24·4
	30	0·767	25·7	33·4
	35	1·36	26·6	19·5
	40	0·831	27·4	33·0
α-Oxoglutarate	10	0·0518	40·2	776
	20	0·0669	55·4	832
	30	0·442	56·1	127
Succinate	10	0·134	45·8	342
	20	0·235	55·4	235
	30	0·800	54·7	68·4
None	10	0·114	—	—
	20	0·156	—	—
	30	0·767	—	—

lase-free purified pyruvate oxidase system (Jagannathan & Schweet, 1952). The pyruvate and α-oxoglutarate oxidase preparations of animal tissues have been shown to contain α-lipoic acid (Schweet & Cheslock, 1952; Sanadi, Littlefield & Bock, 1952). If lipothiamide pyrophosphate was dephosphorylated to lipothiamide and rephosphorylated to the monophosphate, with ATP or inorganic P serving as the source of phosphate, then the subsequent hydrolysis of the amide linkage would result in the formation of radioactive thiamine monophosphate. The reactions postulated are as follows:

(1) cocarboxylase + α-lipoic acid → lipothiamide pyrophosphate;

(2) lipothiamide pyrophosphate → lipothiamide + 2 orthophosphate;

(3) lipothiamide + orthophosphate or ATP → lipothiamide monophosphate;

(4) lipothiamide monophosphate → α-lipoic acid + thiamine monophosphate.

The incorporation of radioactivity into added thiamine monophosphate would be accounted for if reaction (4) occurred in the reverse direction (probably by a different route), but at a much slower rate than the overall rate of reactions (1)–(4), so that the balance of the reactions would favour the formation of free thiamine monophosphate.

SUMMARY

1. The behaviour of thiamine, thiamine monophosphate, cocarboxylase, thiamine triphosphate, oxythiamine, and oxythiamine diphosphate on paper chromatograms is described; *p*-toluenesulphonic acid/*tert.*-pentanol/water was the solvent. The substances are located by spraying with potassium bismuth iodide.

2. Of the thiamine derivatives tested at 0·005 M (thiamine, thiamine monophosphate, cocarboxylase and thiamine triphosphate), only thiamine triphosphate affected the oxidation of pyruvate (40 % inhibition) by sheep-kidney particles; none altered the ratio of the oxygen uptake/pyruvate consumed.

3. Cocarboxylase is converted into thiamine monophosphate by washed suspensions of kidney particles. The amount of cocarboxylase disappearing is less in the presence of pyruvate or α-oxoglutarate than in the absence of substrate, or in the presence of succinate.

4. On incubation of cocarboxylase and ^{32}P-labelled orthophosphate with respiring kidney particles, a slow incorporation of radioactivity into the cocarboxylase occurred. This was unrelated to the amount of substrate oxidized.

5. Thiamine monophosphate formed by breakdown of cocarboxylase in the presence of ^{32}P-labelled orthophosphate was much more radioactive when the tissue suspension contained oxidizable substrate than in the absence of substrate; α-oxoglutarate caused a more rapid incorporation of radioactivity than did succinate.

6. The tissue suspensions were unable to synthesize cocarboxylase from thiamine monophosphate or thiamine, or to synthesize thiamine monophosphate from thiamine; thus it is unlikely that the radioactivity found in thiamine monophosphate was incorporated by rephosphorylation of free thiamine. A mechanism of phosphorylation, assuming that 'lipothiamide' is the reactive form of thiamine is put forward.

7. The steady-state exchange of the phosphate of thiamine monophosphate during the oxidation of α-oxoglutarate has been measured as 0·053 μmole/min.

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The Formation of Phosphopyruvate by Washed Suspensions of Sheep Kidney Particles

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It was observed in previous experiments (Bartley, 1954) that washed, sheep-kidney particles synthesize several phosphate esters when incubated with either pyruvate, succinate or α-oxoglutarate, and ^{32}P-labelled phosphate. One of these compounds is identified in this paper as phosphopyruvate.

METHODS

Phosphopyruvate was prepared by the method of Ohlmeyer (1951). The other substances used, the analytical methods, and the preparation of the tissue suspensions were as previously described (Bartley, 1953; Bartley, 1954).

RESULTS

In the previous experiments (Bartley, 1954), the oxidation of α-oxoglutarate or succinate by suspensions of sheep-kidney mitochondria resulted in the formation of a phosphorylated compound which ran ahead of phosphate on the paper chromatogram when *p*-toluenesulphonic acid:*tert.*-pentanol:water was the solvent ('spot 1'). This substance rapidly became radioactive when the medium contained ^{32}P-labelled phosphate.

Identification of spot 1. On making a paper chromatogram with the above solvent, spot 1 had the same R_F as phosphopyruvate. This was the only phosphate compound of those tested (adenosine phosphates, hexose phosphates, glycerol phosphates, thiamine phosphates, pyrophosphate, pyridoxal phosphate) that had an R_F greater than that of phosphate. The addition of phosphopyruvate to a solution containing spot 1 gave no additional spot with any of the solvents tested (*tert.*-pentanol:formic acid; *p*-toluenesulphonic acid:*tert.*-pentanol:water; *iso*propyl ether:formic acid). In common with phosphopyruvate, spot 1 gave no hydroxamic acid reaction and showed no ultraviolet absorption. The final identification of spot 1 was achieved by determining the phosphate and pyruvic acid content.

A sheep-kidney preparation (to which 0·0025 M thiamine monophosphate was added) was incubated for 30 min. with 60 μmoles of sodium α-oxoglutarate and tracer amounts of ^{32}P-labelled phosphate. The reaction was stopped by the addition of 0·5 ml. of 30% trichloroacetic acid (TCA) and the supernatant solution was used for analysis. Approximately one-third of the total TCA extract (about 2·5 ml.) was put as a band on a 10 cm. line on each of three chromatogram papers, and 50 μl. marker spots were put 2 cm. away from each end of this line. The chromatograms were developed for 20 hr. in a *p*-toluene sulphonic acid:*tert.*-pentanol:water mixture and the papers were then dried at room temperature. The strips of paper bearing the marker spots were cut off and sprayed with molybdate (Hanes & Isherwood, 1949) to locate spot 1. The sprayed strips were laid beside the unsprayed chromatograms and the ends of the bands corresponding to spot 1 were traced. The location of the bands was confirmed and their areas delineated by plotting the areas of radioactivity associated with them. These areas were cut out, and the rest of the paper was sprayed with the molybdate reagent to confirm, from the appearance of the cutting line, that no other phosphorus-containing areas had been included in the excised paper. The material was eluted with water and made up to a volume of 2·3 ml. A 0·2 ml. sample showed no free phosphate when analysed by the method of Berenblum & Chain (1938). Within experimental error, the amounts of phosphorus liberated from 0·2 ml. samples by wet ashing, by hydrolysis for 2 hr. in 1 N-HCl at 100°, or by treatment with alkaline iodine were identical (3·30, 3·40, 3·29 μg.), as were the specific activities of the phosphorus (3130, 3170 and 3030 counts/min./μg. P). This strongly suggests that the material contained one phosphate compound only. Under these conditions phosphopyruvate is also completely hydrolysed.

Of especial significance is the liberation of phosphorus by alkaline iodine, which is considered specific for phosphopyruvate (Lohmann & Meyerhof, 1934). To identify pyruvic acid, a 1·0 ml. sample was heated for 2 hr. at 100° in a stoppered tube with 1·0 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl. A 0·4 ml. sample of this material was analysed for phosphate, and the remainder was shaken with successive 1·0 ml. portions of ethyl acetate to extract the phenylhydrazones. In the combined ethyl acetate extracts, pyruvic acid was determined by the paper ionophoretic method of Neish (1953). A 0·5 ml. sample of the original solution was treated in the same way, omitting the 2 hr. hydrolysis. A standard mixture of pyruvic, α-oxoglutaric and oxaloacetic hydrazones was separated on the same paper as the two test solutions. The ionophoretic separation showed that acid hydrolysis had liberated a substance forming a 2:4-dinitrophenylhydrazone identical with that given by pyruvic acid.

The amount of pyruvic acid liberated was 57% of that expected from the amount of phosphate liberated. A pure sample of phosphopyruvate gave 63% of the theoretical amount of pyruvic acid when treated in the same way.

Conditions of formation of phosphopyruvate. Phosphopyruvate was formed when cocarboxylase, thiamine monophosphate, or thiamine was incubated with a washed suspension of sheep-kidney mitochondria and α-oxoglutarate, succinate, or pyruvate plus bicarbonate. Table 1 shows the amounts formed with these substrates. Phosphopyruvate was not formed from pyruvate when the bicarbonate concentration was below 0·0075M. Thiamine phosphates had been added because the experiments were primarily designed to study the metabolism of these substances. Phosphopyruvate was also formed in the absence of thiamine phosphates. It did not accumulate when the substrate concentration was lowered from 0·0125 to 0·0025M.

Paper chromatography showed that phosphopyruvate was also formed when fumarate, malate and oxaloacetate were the substrates. The quantities were of the same order as in the presence of α-oxoglutarate, succinate, or pyruvate plus bicarbonate, as judged by the colour intensities of the chromatogram.

Incorporation of radioactivity into phosphopyruvate. The rate of incorporation of ^{32}P-labelled phosphate into phosphopyruvate, and the labile phosphate of adenosine triphosphate (ATP) plus adenosine diphosphate (ADP) is shown in Table 2. On short incubation (5–10 min.), the specific activity of phosphopyruvate was much lower than that of ATP and phosphate; but later (after 30–60 min.) the specific activity of all three compounds became equal. The low specific activity of phosphopyruvate at the early stages indicates that phosphopyruvate was not formed directly from phosphate. The data are in accordance with the assumption that ATP is the phosphate donor in the synthesis of phosphopyruvate. The final isotopic equilibrium between ATP, phosphopyruvate and phosphate indicates that the phosphopyruvate formed is being further metabolized; the amount accumulating is thus a measure of the rates of its formation and disappearance. The turnover of phosphopyruvate appears to be very rapid. The addition of 10 μmoles of phosphopyruvate to a system forming phosphopyruvate did not alter the amount of phosphopyruvate (3 μmoles) found after 30 min. incubation, and no phosphopyruvate could be detected after 10 min. incubation when 10 μmoles of phosphopyruvate were added to a kidney suspension which, owing to a low concentration of succinate, does not accumulate phosphopyruvate.

Table 1. *Formation of phosphopyruvate from various substrates in washed sheep-kidney particles*

(Suspension of kidney particles (about 70 mg. dry wt. and 4 ml.) with ATP (0·001M), $MgCl_2$ (0·001M) and orthophosphate containing $^{32}PO_4^{3-}$. 'Phosphopyruvate formed' refers to 4 ml. incubated material.)

Expt. no.	Substrate: Nature	Substrate: Quantity (μmoles)	Ortho-phosphate P added (μmoles)	Thiamine derivative added: Nature	Thiamine derivative added: Quantity (μmoles)	Period of incubation (min.)	Phospho-pyruvate formed (μmoles)
1	Pyruvate + $NaHCO_3$	50·0 + 50·0	30	Cocarboxylase	8·0	60	4·45
2	α-Oxoglutarate	57·6	18·2	Cocarboxylase	8·3	10	1·77
						20	1·33
						30	2·67
3	Succinate	65·6	19·2	Cocarboxylase	6·2	10	0·56
						20	1·04
						30	3·26
4	α-Oxoglutarate	55·7	23·8	Thiamine monophosphate	6·9	5	0·85
						10	0·93
						15	0·47
						20	0·49
						30	2·26
						60	4·44
5	α-Oxoglutarate	39·0	20·5	Thiamine	8·6	35	1·10
						65	2·20

Table 2. *Comparison of specific activities of orthophosphate, labile phosphorus of ATP, and phosphopyruvate*

(Same series of experiments as in Table 1.)

Expt. no.	Substrate: Nature	Substrate: Quantity (μmoles)	Incubation time (min.)	Specific activity (counts/min./μg. P): Ortho-phosphate	Specific activity (counts/min./μg. P): Labile P of ATP + ADP	Specific activity (counts/min./μg. P): Phospho-pyruvate
1	Pyruvate + $NaHCO_3$	50·0	0	670	0	0
		50·0	60	437	408	440
2	α-Oxoglutarate	57·6	0	2088	0	0
			10	1630	1370	530
			20	1260	1050	880
			30	1335	1325	1350
3	Succinate	65·6	0	1655	0	0
			10	1220	780	650
			20	1150	960	1070
			30	1300	1330	1330
4	α-Oxoglutarate	55·7	0	1545	0	0
			5	1430	1080	440
			10	1340	1140	750
			15	1330	1140	750
			20	1320	1290	1020
			30	1330	1210	1050
			60	1270	1200	1270

Table 3. *Formation of phosphate esters by washed kidney particles*

(Initially virtually all phosphate was present as ATP, thiamine phosphates and inorganic phosphate. The amount of phosphate recovered in these three forms after incubation was deducted from the total initial phosphate and is recorded as 'phosphate ester formation'. Same series of experiments as in Table 1.)

Expt. no.	Substrate added	Time of incubation (min.)	Phosphate ester formation (μmoles)	Phosphate ester accounted for as phospho-pyruvate (μmoles)	Phosphate ester unaccounted for (μmoles)
1	Pyruvate + $NaHCO_3$	60	9·64	4·45	5·19
2	α-Oxoglutarate	30	11·37	2·67	8·67
3	Succinate	10	7·57	0·56	7·01
		20	7·95	1·04	6·91
		30	10·36	3·26	7·10
4	α-Oxoglutarate	30	4·50	2·26	2·24
		60	10·38	4·44	5·94
5	α-Oxoglutarate	65	3·90	2·20	1·70

Accumulation of phosphate esters other than phosphopyruvate. As seen in Table 3, phosphopyruvate does not account for all the phosphate that disappears when kidney mitochondria are incubated with α-oxoglutarate, succinate or pyruvate plus bicarbonate. In addition to the phosphopyruvate spot, two, and in some cases three other phosphate-containing spots appear on the paper chromatogram. These have not been identified. Their R_F values are 0·23, 0·3 and 0·5. The first is the spot 4 described previously (Bartley, 1954). The two others do not occur when the pyruvate plus bicarbonate is the substrate. They begin to appear after a lag period of about 20 min. In Expt. 2 (Table 3), the last spot ($R_F = 0·5$) contained about 4 μmoles phosphate after 1 hr., i.e. about the same as the phosphopyruvate spot. The spot of R_F 0·5 reached isotopic equilibrium with the phosphate at about the same time as the phosphopyruvate.

DISCUSSION

The aerobic formation of phosphopyruvate by kidney preparations *in vitro* has been recorded before by Kalckar (1939) (see also Leloir & Muñoz, 1944), who found phosphopyruvate, together with various other organic phosphates, under conditions of incubation similar to those used in the present experiments. Kalckar, too, found no phosphorylation of added pyruvate; phosphopyruvate appeared only when C_4-dicarboxylic acids were added as substrates. The new observation is that the specific

activity of phosphopyruvate is lower than that of inorganic phosphate, and also lower than that of the labile ATP phosphorus during the early stages of the incubation; further, that the turnover of phosphopyruvate is very high. The specific activity data indicate that phosphate does not react directly with the substrate. The low specific activity of phosphopyruvate at the early stages of incubation proves that the phosphate group is derived from a phosphate source other than inorganic phosphate. As ATP is the only alternative source present in sufficient quantities, it must be the phosphate donor. All data are in accordance with this conclusion.

The known pathway of formation of phosphopyruvate from glucose cannot account for the present observations. Lipmann (1941) and Meyerhof (1942) have considered an alternative pathway involving phosphorylation of a C_4-dicarboxylic acid giving phosphoenoloxaloacetate, either directly or via phosphomalate and phosphofumarate. Phosphopyruvate was visualized as arising by decarboxylation from phosphoenoloxaloacetate. This assumption is perhaps at present the most useful working hypothesis, but the earlier schemes have to be modified by replacing ATP for phosphate as the phosphate donor. A direct phosphorylation of pyruvate is feasible under some conditions, as Lardy & Ziegler (1945) have demonstrated, showing the reversibility of the reaction

$$\text{phosphopyruvate} + \text{ADP} \rightleftharpoons \text{pyruvate} + \text{ATP}. \quad (1)$$

But the equilibrium constant of this reaction at pH 7·0 is about 7×10^{10} (see Meyerhof & Oesper, 1949; Burton & Krebs, 1953). Therefore no measurable quantities of phosphopyruvate could accumulate under the conditions of the present experiments. This is borne out by the fact that pyruvate, alone, without bicarbonate, does not yield phosphopyruvate.

The significance of the formation of phosphopyruvate remains to be established. It cannot be a step in oxidative phosphorylation, but it is plausible that the synthesis of phosphopyruvate, by a reaction other than the reversal of reaction (1), is a key step in the resynthesis of glucose from C_4 and C_3 compounds. Isotope data on the role of bicarbonate in the synthesis of glycogen (Solomon, Vennesland, Klemperer, Buchanan & Hastings, 1941) are in accordance with this concept.

SUMMARY

1. Phosphopyruvate is formed by washed, sheep-kidney particles during the oxidation of α-oxoglutarate, succinate, fumarate, malate, oxaloacetate, and pyruvate plus bicarbonate; it is not formed during the oxidation of pyruvate alone. This is in accordance with earlier observations of Kalckar (1939). Phosphate esters other than phosphopyruvate are formed during the later stages of the incubation.

2. After short periods the specific activity of phosphopyruvate is lower than that of phosphate. The data exclude phosphate, and suggest adenosine triphosphate as the immediate precursor of the phosphate group of phosphopyruvate.

3. The observations that phosphopyruvate rapidly reaches isotopic equilibrium with added $^{32}PO_4^{3-}$ and that added phosphopyruvate is rapidly removed indicates that the turnover rate of phosphopyruvate is high.

4. The mechanism of formation of phosphopyruvate is discussed. It is suggested that reactions of the type proposed by Meyerhof (1942) and by Lipmann (1941), involving C_4-dicarboxylic acids, are probably implicated. The formation of phosphopyruvate may be a step in the synthesis of carbohydrate.

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The Reduction of Sulphate in the Rumen of the Sheep

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The micro-organisms in the rumen of the sheep are capable of reducing nitrate and nitrite to ammonia (Sapiro, Hoflund, Clark & Quin, 1949; Lewis, 1951*a*, *b*). It was thought desirable to investigate whether this mixed population could also carry out the analogous reduction of sulphate to hydrogen sulphide, particularly since sulphate is a significant constituent of grass and hay, the normal foodstuff of ruminants. It has been shown by Thomas, Hendricks & Hill (1950) and by Evans (1931) that the normal concentration of sulphate in grass is of the order of 0·3 % sulphate S in the dry matter, rising under certain circumstances to 1 %. On the basis of a daily intake of 1 kg. dry matter, this is equivalent to 13 and 44 g., respectively, of Na_2SO_4 each day.

Beijerinck (1895) showed that the reduction of sulphate to sulphide in mud was a biological process, and van Delden (1904) isolated an organism, *Vibrio desulphuricans*, which would carry out this process. The organism was a strict anaerobe and it was found that the reduction of sulphate was linked with the oxidation of a variety of organic compounds (see also Baars, 1930). Stephenson & Stickland (1931) isolated from river mud a sulphate-reducing organism which would use gaseous hydrogen as well as organic substances as a hydrogen donor.

The isolation and cultivation of sulphate-reducing bacteria have been described in detail by Butlin, Adams & Thomas (1949). They consider that there is sufficient evidence to assume the existence of several species. Postgate (1951*a*, *b*; 1952) has studied in detail the nutrition and metabolism of *Desulphovibrio desulphuricans*; he used washed suspensions in Warburg manometer vessels for the study of the metabolism of this organism. It was able to utilize sulphate, thiosulphate, sulphite and tetrathionate for growth and its resting metabolism; it was also apparently able to use dithionite and metabisulphite. The author points out that these compounds are not stable. It was considered that the simplest interpretation of the evidence available was that the normal sequence of reactions was as follows:

$$SO_4^{2-} \xrightarrow{H_2} SO_3^{2-} \rightarrow \begin{matrix}(\text{unknown})\\(\text{intermediates})\end{matrix} \rightarrow S^{2-}.$$

Information on sulphate metabolism in the ruminant is limited. Block, Stekol & Loosli (1951) have demonstrated that labelled sulphate fed to the goat is rapidly incorporated into the cystine and methionine of the milk proteins, but no information is available on the mechanism of this conversion. Evidence is already available (Lewis, unpublished) that sulphide is released from L-cysteine by washed suspensions of rumen micro-organisms. It is therefore possible that in the rumen the sulphur-containing amino acids give rise to sulphide and that part at least of the sulphate ingested is converted into sulphide. It would thus seem likely that the bulk of the sulphur supply of the ruminant is at one stage in the form of sulphide. The sulphide produced from sulphate or from sulphur-containing amino acids may be either absorbed from the rumen or the other parts of the alimentary canal into the blood stream, or it may be excreted in the faeces or resynthesized into the sulphur-containing amino acids of the proteins of micro-organisms in the rumen. Thus, information on the production of sulphide in the rumen was thought necessary in order to understand the normal sulphur metabolism of the ruminant and having regard to any possible abnormality or toxicity caused by a high concentration of sulphide.

The present work has been restricted to a study of the reduction of sulphate by rumen micro-organisms. The reduction *in vivo* was investigated by the introduction of sulphate into the rumen through a permanent rumen fistula. The reduction of sulphate *in vitro* was studied with washed suspensions of rumen organisms.

METHODS

Design of animal experiments. The experimental animals were fitted with permanent rumen fistulae: three Scotch blackface wethers at Sheffield and two Clun Forest wethers at Babraham. The Scotch blackface wethers weighed approx. 40 kg. and were fed 230 g. Paul's no. 2 dairy nuts (N, 3·1 %) daily, plus a mixture of chopped hay and straw *ad lib.* The others weighed approx. 60 kg. and were fed 1 kg. of chopped hay daily. During the course of the *in vivo* experiments the sheep were fed at between 9.00 and 9.30 a.m. and the excess of foodstuff was removed 2 hr. later. At this point the initial sample of rumen contents was withdrawn through the fistula. A second sample was withdrawn 1 hr. later and the solution containing a known amount of sodium sulphate was introduced into the rumen.

Succeeding samples were withdrawn at the appropriate times. When the sheep were regularly dosed with sulphate, an aqueous solution of Na_2SO_4 was placed in the rumen through the fistula 2 hr. after feeding commenced. The sheep were not dosed on the days when animal experiments were carried out. The *in vivo* experiments reported were carried out at Babraham and those *in vitro* at Sheffield.

Washed suspensions of rumen bacteria were prepared according to the method of Sijpesteijn & Elsden (1952), save that no Na_2S was added to the washing solution. The sample of rumen contents was withdrawn 5–5·5 hr. after feeding. The activity did not appear to be affected by the omission of the sulphide, provided that the following precautions were adhered to. Oxygen was removed as far as possible from the reagents, including the phosphate buffer (K salts in all cases) by boiling for 2 min., cooling rapidly and gassing with a slow stream of O_2-free N_2 until used. The substrates were not boiled. Unless otherwise stated, all washed suspensions were prepared from rumen contents obtained from sheep that had been previously dosed with Na_2SO_4 for at least 14 days.

Manometric methods. The reactions were carried out at 37° in Warburg manometers with double side-bulb cups. The main compartment contained 2 ml. of the washed cell suspension and the first side bulb contained the potential H-donor and the sulphate. The contents of this side bulb were tipped in when thermal equilibrium had been attained. The second side bulb contained 0·4 ml. 4 N-H_2SO_4 which was tipped in at the end of the incubation period to stop the reaction and to release any H_2S which had been bound by the medium. Failure to do this led to low recoveries of sulphide. The centre wells contained 0·2 ml. 20% (w/v) NaOH and a plug of acid-washed glass wool to trap any sulphide produced and to absorb any CO_2 formed. Glass wool was used in preference to filter paper, since the latter disintegrated in the presence of alkali.

Total nitrogen. This was determined by the Kjeldahl method as modified by Chibnall, Rees & Williams (1943) using a Markham (1942) steam-distillation apparatus and the boric acid reagent of Conway & O'Malley (1942).

Sulphide estimation. Sulphide was determined by a colorimetric method developed by Wilkinson & Fry (1950). It is based on the reaction of H_2S with I_2 in acid solution:

$$H_2S + I_2 \rightarrow 2HI + S.$$

A solution of I_2 in CCl_4 is purple in colour (maximum absorption at 520 mμ.) and Fig. 1 shows that Beer's law is obeyed over the range of 0–0·0007 M. When a solution of I_2 in CCl_4 is shaken with an acidified aqueous solution of H_2S, the I_2 reacts with the sulphide and the depth of colour in the CCl_4 is reduced. Thus, from a determination of the decrease in optical density after treatment with a solution containing H_2S, it is possible to obtain an estimate of the amount of sulphide present. This method has been used for the determination of sulphide produced in biological systems.

The sulphide produced in the manometer vessels was absorbed by alkali in the centre well. At the end of the period of incubation, the acid was added from the side bulb and the manometer shaken for a further period of 30 min. The plug of glass wool was carefully transferred with forceps, afterwards washed, into a separating funnel. The total volume at this stage was 3 ml. To the separating funnel were added 2 ml. of 0·005 M-I_2 in redistilled CCl_4, 3 ml. CCl_4 and 5 ml. 2 N-HCl. The I_2 solution had been previously standardized by shaking with a known excess of aqueous

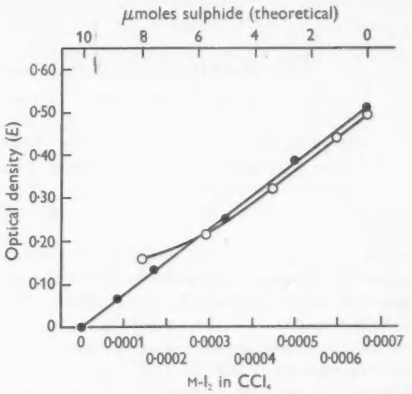

Fig. 1. Optical density of iodine in carbon tetrachloride. ●, dilution of 0·00503 M-I_2 in CCl_4; ○, addition of known amounts of sulphide to 2 ml. 0·00503 M-I_2 in CCl_4. Volume made up to 15 ml. with CCl_4. For conditions see text.

$Na_2S_2O_3$ and back-titrating with a standard solution of I_2 prepared from potassium bi-iodate. After being shaken vigorously for 2 min., the CCl_4 layer was run into a 15 ml. measuring flask and the contents of the separating funnel were washed twice by shaking with 3 ml. volumes of CCl_4. Care was taken to avoid evaporation by keeping all vessels stoppered. The volume was made up to 15 ml. with redistilled CCl_4 and the optical density measured in an EEL (Evans Electroselenium Ltd., Harlow, Essex) colorimeter with a green filter.

The sulphide concentration in rumen contents was estimated using Conway units (Conway, 1950). In the outer compartment of the dish were placed 2 ml. of rumen contents which had been filtered through muslin, and in the inner compartment 1 ml. 5% (w/v) NaOH; the dishes were then covered and 0·4 ml. 4 N-H_2SO_4 was added to the outer compartment as described by Conway (1950). The vessels were allowed to stand overnight at room temperature and the alkali was transferred, with washing, to a separating funnel. The procedure thereafter was the same as described above.

Fig. 1 shows the relationship between the optical density of the I_2 solution and μmoles sulphide added. Within the range of 0–6 μmoles sulphide added to 2 ml. 0·005 M-I_2 in CCl_4, this was linear. With larger amounts of sulphide, however, it appeared that the reaction was no longer quantitative. For the purpose of this experiment, a solution of Na_2S was used which had been standardized directly against an I_2 solution prepared from potassium bi-iodate. The difference between the standard I_2 curve and that following the addition of sulphide may be due to mechanical losses of I_2 in the manipulations. The recoveries of sulphide added to washed suspensions of rumen contents in Warburg manometer vessels and Conway dishes were also determined. A representative selection of the results is presented in Table 1. A standard curve was prepared for each series of estimations by dilution of 0·005 M-I_2 in CCl_4 solution. The figures of apparent recovery of sulphide from 2 ml. of distilled water probably refer to losses of the I_2 in CCl_4

Table 1. *Recovery of sulphide added to rumen contents*

(The observed values are corrected for the mean of duplicate estimations of sulphide in rumen contents.)

Sulphide added (μmoles)	Fluid	Recovered from Conway dishes			Recovered from Warburg manometers		
		μmoles	μmoles (corr.)	%	μmoles	μmoles (corr.)	%
0	2 ml. water	0·20	—	—	0·18	—	—
0		0·18	—	—	0·17	—	—
0	2 ml. RC*	0·74	—	—	0·66	—	—
0		0·69	—	—	0·73	—	—
1·13		1·83	1·11	98	1·89	1·19	105
1·13		1·88	1·16	103	1·74	1·04	92
3·39		4·11	3·39	100	3·95	3·25	96
3·39		4·15	3·43	101	3·86	3·16	93
5·65		5·82	5·10	90	5·95	5·25	93
5·65		5·97	5·25	93	5·78	5·08	90
7·91		7·19	6·47	82	7·20	6·50	82
7·91		7·11	6·39	81	7·38	6·68	84

* RC = Rumen contents after filtering through muslin.

solution. The quantity recovered from rumen contents appears to fall off with larger amounts of added sulphide. The method has been used only within the range of 1–6 μmoles sulphide, where the recoveries were always within the range 90–105%.

Sulphate estimation. Sulphate was determined by a modification of the standard, gravimetric $BaSO_4$ procedure (Vogel, 1951). A volume of 200 ml. rumen contents was withdrawn and filtered through muslin. Duplicate 50 ml. samples of this were used for analysis. A suitable volume of 2 N-HCl was added to give a final concentration of 0·01 N added HCl and after being stirred, the whole was centrifuged at 3600 rev./min. for 30 min. The residue was washed with 50 ml. 0·01 N-HCl and spun again. The combined supernatants were heated to 100° and 25 ml. 5% (w/v) $BaCl_2.2H_2O$ added. After being kept in a boiling-water bath for 20 min., the precipitate was filtered through a weighed, sintered-glass crucible (no. 4), washed, dried and weighed again. Recoveries of 200–1200 μmoles Na_2SO_4 added in aqueous solution (weighed out as $Na_2SO_4.10H_2O$) to the 50 ml. samples of filtered rumen contents, were always within the range of 95–101% after subtracting the blank concentration in rumen contents which was always less than 1·5 μmoles sulphate/ml. This was composed of sulphate and some undefined materials precipitated by the $BaCl_2$.

RESULTS

Reduction in the rumen

In order to determine whether sulphate is reduced in the rumen of the sheep, the concentrations of sulphate and sulphide were determined following the introduction of sulphate into the rumen. If the sheep had not been previously dosed with sulphate, the sulphate added disappeared at a steady rate and some sulphide was always formed, but both the amount of sulphide formed and the rates at which it was produced and disappeared were very variable. The results of a typical experiment are presented in Fig. 2, including those of a control experiment in which no sulphate was introduced into the rumen.

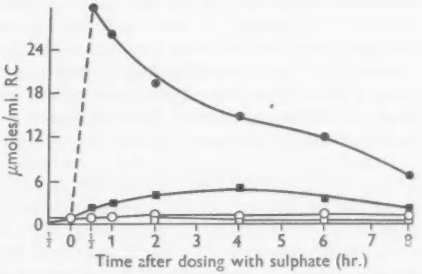

Fig. 2. Sulphate and sulphide in the rumen contents (RC) following dosing with sulphate. Dose, 80 g. $Na_2SO_4.10H_2O$ (41·4 μmoles/ml. RC, assuming the rumen has a capacity of 6 l.). Control, no dose administered. □, control sulphide; ○, control sulphate; ■, experimental sulphide; ●, experimental sulphate.

The concentration of sulphide in the rumen in the absence of added sulphate is consistently low and usually less than 0·8 μmole/ml. rumen contents. It can be seen that there is a rapid disappearance of added sulphate; within 4 hr. the concentration is less than half that found 0·5 hr. after dosing. Within 24 hr. the concentration had fallen to the blank level of 0·5 μmole/ml.

Experiments were also carried out after the sheep had been dosed daily with 40 g. $Na_2SO_4.10H_2O$ in 200 ml. water. The daily dose was administered 2 hr. after feeding, and 24 hr. later the concentration of both sulphate and sulphide in the rumen had returned to the blank level. The results of one such experiment are given in Fig. 3, after the sheep had been dosed for 14 consecutive days. The usual dose was not administered on the day that the experiment was carried out. In this experiment the disappearance of sulphate was more rapid and the concentration of sulphide obtained was greater. The

concentrations of sulphate in the rumen contents 4 hr. after the test substance had been administered were 14·7 μmoles (no pre-dosing) and 6·0 μmoles (after 14 doses)/ml. and the sulphide concentrations at the same time were 4·8 and 8·5 μmoles/ml., respectively.

In order to determine whether there was a significant increase in the rate of sulphate disappearance from the rumen when the sheep had been dosed daily with sulphate, a series of experiments was carried out before and after dosing the sheep. The results are expressed as the time taken to decrease the concentration of sulphate in the rumen by a half. The curve of sulphate concentration is extrapolated to the time of dosing, and the time after dosing at which the concentration is halved is recorded. These semiquantitative data for a series of experiments are presented in Table 2; the differences are consistent and probably significant. The results of these *in vivo* studies show that sulphate is reduced to sulphide in the rumen of sheep and that the rate of sulphate disappearance and sulphide production is increased by the repeated dosage of the sheep with sulphate. No information is available about the quantity of sulphate absorbed from the rumen nor the amount that passes along the alimentary tract.

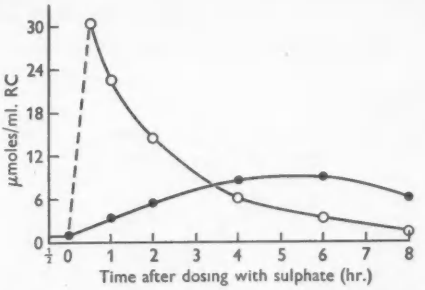

Fig. 3. Sulphate and sulphide in the rumen following repeated dosing of a sheep with sodium sulphate. Sheep given daily dose of 40 g. $Na_2SO_4.10H_2O$ for 15 days, and experimental dose of 80 g. $Na_2SO_4.10H_2O$ at time 0. ○, sulphate; ●, sulphide.

Table 2. *Disappearance of sulphate from the rumen of the sheep*

(The animals dosed with sulphate were given a solution of 40 g. $Na_2SO_4.10H_2O$ daily, and the experimental dose consisted of 80 g. $Na_2SO_4.10H_2O$ in 250 ml. water given 3 hr. after feeding.)

	Time for reduction of sulphate to half of the initial concentration (hr.)	
	Sheep not dosed	Sheep dosed on 14 successive days
Sheep 8	4·2	1·4
Sheep 8	2·6	1·2
Sheep 11	4·3	1·7
Sheep 12	2·5	1·3
Sheep 8	3·2	2·5

Washed-suspension experiments

The reduction of sulphate with gaseous hydrogen as hydrogen donor. The ability of washed suspensions of rumen micro-organisms to reduce sulphate with gaseous hydrogen as the hydrogen donor was tested. When sulphate was incubated with the washed cells in an atmosphere of hydrogen, there was a steady uptake of hydrogen greater than that observed in the control manometer which contained no sulphate (Fig. 4). An additional control was arranged in which sulphate was incubated with the suspension under an atmosphere of nitrogen. The first control was used for the correction of the observed hydrogen uptake and the second for the correction of the figures of sulphide formation. Hydrogen sulphide was estimated in the alkali withdrawn from the centre wells and the results are presented in Table 3. The hydrogen uptake corresponded to 111 and 114 % (not corrected) and 89 and 92 % (corrected) of the theoretical value for reduction to sulphide: the figures for sulphide production were 100 and 104 %, and 74 and 78 %, respectively. Despite the apparently good agreement with the uncorrected values, it is thought that the figures after the subtraction of the blanks are probably more reliable, since almost half of the sulphide in the blank can be accounted for in terms of that initially present in the washed suspension. It was found that the sulphide content of the suspension was not markedly affected by the washing procedure. The fact that the amount of hydrogen used, even correcting for the control, is

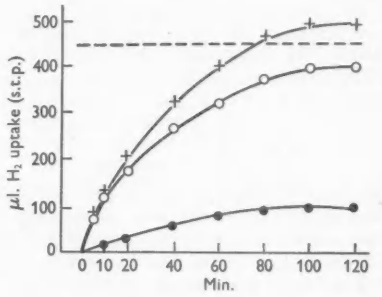

Fig. 4. Hydrogen uptake by washed micro-organisms in the presence of sulphate. Theoretical uptake is for reduction to sulphide. 2 ml. washed-cell suspension in 0·1 M potassium phosphate buffer (pH 6·5), containing 16·4 mg. total N/100 ml.; 0·1 ml. 0·05 M-Na_2SO_4 in side bulb; 0·2 ml. 20 % (w/v) NaOH in centre well. ●, control; +, observed; ○, corrected.

greater than the sulphide produced suggests that hydrogen is being used in some other reaction.

Reduction of other inorganic sulphur compounds. The ability of the washed suspensions in an atmosphere of hydrogen to reduce sulphite, dithionite, and thiosulphate, in the presence of hydrogen, was next examined. There was a rapid uptake of hydrogen in the presence of sulphate, thiosulphate and sulphite, but not when dithionite was added (Fig. 5).

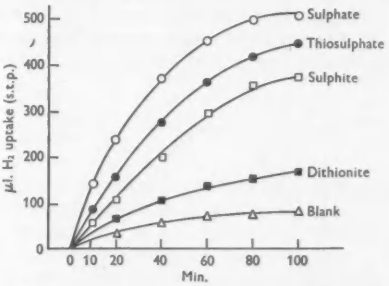

Fig. 5. Hydrogen uptake in the presence of sulphate, sulphite, dithionite and thiosulphate. 2 ml. Suspension in 0·1 M phosphate buffer (pH 6·5) containing 19·4 mg. total N/100 ml.; 0·2 ml. 0·025 M substrate in side bulb; 0·2 ml. 20% (w/v) NaOH in centre well. △, control; ■, sodium dithionite; □, sodium sulphite; ●, sodium thiosulphate; ○, sodium sulphate.

At the end of the 100 min. incubation period, the acid was tipped in from the side arm. The sulphide production was estimated; the results, together with theoretical values for reduction to sulphide, are presented in Table 4. The appropriate corrections have been made; the control values were of the same order as those in Table 3. There was clearly a reduction of sulphate, sulphite, and thiosulphate, to sulphide, with a hydrogen uptake and sulphide production within the range of 70–100% of the theoretical. In the presence of dithionite it is doubtful whether there was a significantly greater hydrogen uptake or sulphide production than in the control. Postgate (1951*b*) has shown that *D. desulphuricans* is able to utilize sulphate, thiosulphate, sulphite and tetrathionate for its growth and resting metabolism. However, though it was also apparently able to use dithionite and metabisulphite, the author considers the results to be of doubtful significance because of the spontaneous decomposition of these compounds.

The effect of pH *on the reduction of sulphate by washed suspensions in the presence of hydrogen.* The preparation of the washed-cell suspension differed in that the rumen micro-organisms were both washed with and suspended in a saline prepared as follows: a solution of 0·9% (w/v) NaCl was boiled immediately before use and cooled rapidly under the tap; to this were added 5% aqueous thioglycollic

Table 3. *Hydrogen uptake and sulphide formation by washed suspension in the presence of sulphate*

(2 ml. washed-cell suspension in 0·1 M phosphate buffer (pH 6·5) containing 19·8 mg. total N/100 ml.; 0·1 ml. 0·05 M-Na_2SO_4 (5 μmoles) in first side bulb: 0·4 ml. 4 N-H_2SO_4 in second side bulb: 0·2 ml. 20% (w/v) NaOH in centre well. Theoretical values are for reduction to sulphide.)

		H_2 uptake			Sulphide formation		
Substrate	Gas phase	Obs. (μmoles)	Corr. (μmoles)	% Theory corr.	Obs. (μmoles)	Corr. (μmoles)	% Theory corr.
Na_2SO_4	H_2	22·2	17·8	89	5·0	3·7	74
Na_2SO_4	H_2	22·8	18·4	92	5·2	3·9	78
Na_2SO_4	N_2	0·3*	—	—	1·2	—	—
Na_2SO_4	N_2	0·4*	—	—	1·4	—	—
None	H_2	4·6	—	—	0·6	—	—
None	H_2	4·3	—	—	0·9	—	—

* An apparent uptake of gas that may have been due to traces of O_2 in the N_2 used.

Table 4. *Reduction of inorganic sulphur compounds to sulphide*

(2 ml. suspension in 0·1 M phosphate buffer (pH 6·5) containing 19·4 mg. total N/100 ml.; 0·2 ml. 0·025 M sodium salt of substrate in first side bulb (5 μmoles); 0·2 ml. 20% (w/v) NaOH in centre well; 0·4 ml. 4 N-H_2SO_4 in second side bulb. Theoretical results are for reduction to sulphide and figures have been corrected for appropriate blanks.)

	H_2 uptake			Sulphide production		
	Theory (μmoles)	Found (μmoles)	% Theory	Theory (μmoles)	Found (μmoles)	% Theory
Control (N_2/SO_4^{2-})	—	0	—	—	0·9	—
Control (H_2 only)	—	3·8	—	—	0·4	—
Sulphate	20	18·9	95	5	3·6	72
Sulphite	15	13·0	87	5	4·1	82
Thiosulphate	20	16·2	81	10	7·5	75
Dithionite	25	3·8	15	10	2·3	23

acid to a final concentration of 0·02% (w/v) and sufficient 0·1N-NaOH to bring the pH to 6·5. The main compartment of the manometer vessels contained 1 ml. of the suspension and 1 ml. 0·2M potassium phosphate buffer, of the appropriate pH, which had also been boiled and cooled rapidly and which contained 0·02% (w/v) thioglycollic acid. The buffers covered the range of pH 5·8–8·0. A control manometer containing no substrate was set up at each pH. The sulphate (0·2 ml. 0·05N) was tipped in from a side bulb after a period of equilibration. Initial rates of hydrogen uptake, from 5 to 10 min. after tipping in the substrate, were determined and corrected for the gas uptake in the controls; the blanks were of the order of 15–20% of the values in the presence of the substrate. The results (Fig. 6) are expressed as percentages of the maximum initial rate of hydrogen uptake, and show that the optimum pH for sulphate reduction by the washed rumen micro-organisms is around 6·5.

Hydrogen donors other than gaseous hydrogen. The compounds tested are either known to occur in the rumen or are thought to be present under certain conditions. For convenience the experiments were carried out in Warburg manometer vessels in an atmosphere of nitrogen. In addition to the control in which sulphate was incubated with the suspension in the absence of hydrogen under nitrogen, a second manometer was set up in which the hydrogen donor was hydrogen gas. This latter served as a reference standard whereby the efficacy of the hydrogen donors under examination could be judged. The test substances were incubated in the presence of sulphate and the washed suspension for 2 hr. and the ability of the compounds to act as hydrogen donors was assessed in terms of the amounts of sulphide produced. It was considered that a significant reduction of sulphate was effected when the sulphide produced in the presence of the potential hydrogen donor was at least double that in the control. In no case did the reaction go to completion in the 2 hr. incubation period. There was a significant reduction of sulphate in the presence of hydrogen, glucose, fructose, DL-lactate, pyruvate, formate, succinate, ethanol, citrate and L(−)-malate but not in the presence of the fatty acids, D-xylose, mannitol or glycerol (Table 5).

Fractions of rumen micro-organisms. The experiments *in vivo* show that dosing the sheep with sulphate increases the rate of sulphide production from sulphate. In view of this, a series of experiments was designed to determine whether dosing the sheep with sulphate affects the rate of sulphate reduction by fractions of washed-cell suspensions of rumen micro-organisms. Each of the fractions was suspended in 0·1M phosphate buffer (pH 6·5) in a volume equivalent to one-third of that initially withdrawn from the rumen. At the time of the first experiment (expt. 1) the sheep had not been dosed with sulphate for 28 days: after this, a dose of 40 g. $Na_2SO_4.10H_2O$ in 200 ml. water was introduced daily into the rumen of the sheep except on the days when a sample was withdrawn for investigation. Succeeding experiments were carried out on the third day (expt. 2), the 6th day (expt. 3) and the 18th day (expt. 4) after the commencement of the daily dosing of the sheep. The separation of the fractions was effected on a M.S.E. 'Angle' centrifuge (Measuring and Scientific Equipment Ltd., London, S.W. 1). The sample of rumen contents was filtered through muslin and spun at 700 rev./min. for 2 min.

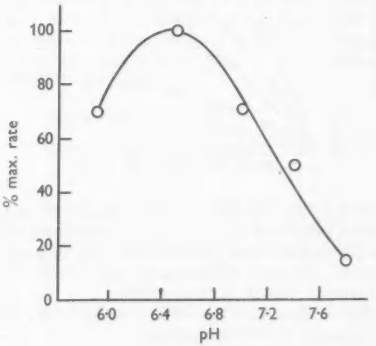

Fig. 6. Effect of pH on rate of hydrogen uptake by washed cell suspensions in presence of sulphate. Rates expressed as % of the maximum initial rate of hydrogen uptake. Suspension containing 25·4 mg. total N/100 ml. For details see text.

Table 5. *Production of sulphide from sulphate by washed suspensions of rumen bacteria in presence of potential hydrogen donors*

(2 ml. washed-cell suspension in 0·1M phosphate buffer (pH 6·5); 0·4 ml. 0·1M H-donor; 0·2 ml. 0·1M-Na_2SO_4 (20 μmoles) in first side bulb and 0·4 ml. 4N-H_2SO_4 in second side bulb; 0·2 ml. 20% (w/v) NaOH in centre well. Incubation for 2 hr. in N_2 atmosphere.)

H-donor	μmoles sulphide	H-donor	μmoles sulphide
Expt. A		Expt. C	
Control	2·1	Control	1·2
Hydrogen	15·4	Hydrogen	15·3
Glucose	16·1	Acetate	1·6
Fructose	13·9	Propionate	1·2
DL-Lactate	12·9	Butyrate	1·8
Pyruvate	11·2	Valerate	2·1
Expt. B		Expt. D	
Control	3·7	Control	1·9
Hydrogen	18·9	Hydrogen	16·3
Formate	16·2	Citrate	8·2
Succinate	11·1	D-Mannitol	3·2
Ethanol	10·0	Glycerol	3·7
D-Xylose	4·2	L(−)-Malate	7·0

Table 6. *Rate of sulphide production and hydrogen uptake by fractions of rumen micro-organisms (see text) in the presence of sulphate at different stages of a course of daily dosing with sulphate*

(2 ml. suspensions: total N approx. 8 mg. (residue 1), 6 mg. (residue 2) and 4 mg. (residue 3). 0·3 ml. 0·02 M-Na_2SO_4 in first side bulb; 0·4 ml. 4 N-H_2SO_4 in second side bulb; 0·2 ml. 20% (w/v) NaOH in centre well. Incubation for 30 min. under H_2 atmosphere.)

	Expt. 1 (before dosing)		Expt. 2 (3rd day of dosing)		Expt. 3 (6th day of dosing)		Expt. 4 (18th day of dosing)	
	Obs.	Corr.	Obs.	Corr.	Obs.	Corr.	Obs.	Corr.
	μmoles H_2S/mg. total N/hr.							
Residue 1	0·2	0·1	0·7	0·4	0·6	0·5	0·8	0·6
Residue 2	0·4	0·2	1·1	0·7	1·5	1·1	1·8	1·5
Residue 3	0·9	0·5	2·0	1·5	3·3	2·5	3·0	2·4
	μmoles H_2/mg. total N/hr.							
Residue 1	0·5	0·2	1·8	1·2	1·2	0·8	3·4	2·5
Residue 2	1·0	0·4	4·7	2·8	4·9	3·8	6·4	4·8
Residue 3	2·6	1·2	9·1	7·1	10·3	8·0	11·9	10·0

(residue 1); the supernatant was spun at 1900 rev./min. for 5 min. (residue 2); and in the same way at 3550 rev./min. for 25 min. (residue 3). The residues were then washed with and suspended in the 0·1 M phosphate buffer (pH 6·5) prepared as before but containing no thioglycollic acid.

The manometer vessels contained 2 ml. of these suspensions, together with 6 μmoles Na_2SO_4 in an atmosphere of hydrogen, with alkali in the centre well. The incubation was stopped after 30 min., the hydrogen uptake was recorded and the sulphide absorbed in the alkali was estimated. Control manometers were arranged in each instance in the absence of sulphate (for the correction of the hydrogen uptake) and in the presence of sulphate in nitrogen atmosphere (for correction of sulphide production). The results (Table 6) are expressed in the form of metabolic quotients, as μmoles H_2 or H_2S/mg. total N/hr. The calculations were made on the basis of the gross uptake of hydrogen or production of sulphide in the 30 min. incubation period. Corrections have also been made for the blank reactions. This form of expression was considered desirable since it compensated for differences in the total nitrogen of the suspensions, but it must be pointed out that the hydrogen-uptake curves were not strictly linear throughout the 30 min. incubation. In no instance did the reaction go to completion. The maximum value of $Q_{H_2}^{H_2}$ recorded was an uptake 266 μl. H_2/mg. total N/hr., which may be compared with a $Q_{H_2}^{H_2}$ (sulphate) of 200 on a dry-weight basis found by Postgate (1951*b*).

In all cases there was a more rapid reduction of sulphate by the fraction containing the smaller organisms (residue 3). The rate was almost invariably more than twice as great as with residue 2. With some exceptions the sulphide production was greater than expected in view of the hydrogen uptake, suggesting that in these instances other hydrogen donors were present to some extent in the suspension. The relationship between hydrogen uptake and sulphide production does not always agree well with the expected 4 moles H_2 taken up/mole sulphide formed. It was also clearly shown that dosing of the sheep with sulphate encouraged the rate of sulphide production.

DISCUSSION

It has been shown that sulphate is reduced to sulphide by the rumen micro-organisms both *in vivo* and *in vitro* using washed suspensions of rumen micro-organisms. There is no reason to believe that the presence of sulphate in any way stimulates the release of sulphide from such compounds as the sulphur amino acids. The parallel findings *in vivo* and *in vitro* present a further confirmation of the reliability of the washed-suspension method, although the objections listed by Sijpesteijn & Elsden (1952) remain. The pH optimum of the reaction, about 6·5, is about the normal pH of rumen contents (Phillipson, 1942). A similar pH optimum was shown by Lewis (1951*b*) for the reduction of nitrate, nitrite and hydroxylamine, and by Sijpesteijn & Elsden (1952) for the decarboxylation of succinic acid. Postgate (1949) stated that the optimum pH for sulphate reduction by *D. desulphuricans* with hydrogen gas as hydrogen donor is 6·3, confirming the results of Stephenson & Stickland (1931). Butlin *et al.* (1949) recommend that these bacteria be grown at pH 7·2. Postgate (1951*a*) showed that *D. desulphuricans* grew well from pH 6·3 to 8·6, growth being more rapid at the higher pH, a feature attributed to the concentration of bicarbonate in the medium.

The hydrogen donors found to be active for sulphate reduction by the washed suspensions are similar to those found to be effective in nitrate reduction (Lewis, 1951*b*). There is no evidence available to indicate whether or not these are the

compounds effective for sulphate reduction in the rumen. The evidence for their occurrence in the rumen is very limited; only the fatty acids have been shown to be present in significant quantities and these are not active hydrogen donors. In both the nitrate and the sulphate reduction, hydrogen gas was amongst the most active hydrogen donors; in a careful analysis of rumen gases, Lugg (1938) was not able to demonstrate the presence of hydrogen.

The reduction is presumed to be carried out by sulphate-reducing bacteria in the rumen, but the organism or organisms responsible have not been isolated. Several of the properties of the washed suspension of rumen micro-organisms are similar to those of *D. desulphuricans*. A comparison may be made of the inorganic sulphur compounds reduced (cf. dithionite), the effective hydrogen donors (Baars, 1930), the strict anaerobiosis required, and the pH optimum. The presence of sulphate-reducing organisms is strongly suggested by the results of dosing the sheep with sulphate; the more rapid reduction *in vivo* is interpreted as being due to the rapid proliferation of the organisms in a sort of 'enrichment' medium. A parallel enhanced rate of reduction was demonstrated *in vitro*. It was also shown that the fractions containing the smaller organisms have the greater ability to reduce sulphate. None of these results contradicts the idea that the reduction is brought about by organisms resembling *Desulphovibrio*.

Preliminary efforts to isolate sulphate-reducing organisms from the rumen have not been successful. It is, however, desirable that this be carried out. An attempt was made to assess the number of such organisms present by the technique of including ferrous sulphate in a solid agar medium, so that in the vicinity of a sulphate-reducing organism the sulphide gives rise to a black spot. The results obtained were, however, uncertain and in some cases no colonies were observed even after 14 days incubation. Tentative figures obtained were of the order of 10^3 organisms/ml. of rumen contents in the sheep not dosed with sulphate, and 10^5 organisms/ml. following the dosing of the sheep for 14–28 days. It is realized that the results were variable, that the figures found were low and also that the technique does not exclude the possibility of sulphide production from sulphur-containing amino acids. The results suggested an increase in numbers following dosage. It must be pointed out that there have been certain inconsistencies in the results of the experiments on sulphate reduction with the techniques used. During some *in vivo* experiments the quantity of sulphide produced was very low, the washed suspensions sometimes did not appear to reduce sulphate, particularly if the sheep had not been previously dosed with sulphate, and often there were no demonstrable colonies in the solid agar medium when the method described above was used (see Butlin *et al.* 1949).

Since hydrogen sulphide is toxic, the possibility that diets rich in sulphate might exert toxic effects had to be considered. However, when a dose of 150 g. $Na_2SO_4.10H_2O$ was given, no toxic effects were observed even though the concentration of sulphide in the rumen reached 14·7 μmoles/ml. rumen liquor. Assuming the normal and maximum concentrations of sulphate in the dry matter of the diet to be 0·3 and 1 % sulphate S, respectively, and a daily intake of 1 kg. dry matter, this is equivalent to an intake of 30 and 100 g., respectively, of $Na_2SO_4.10H_2O$. So at this range no gross abnormality or toxicity can be expected.

However, the sulphide produced in the rumen probably contributes to the normal sulphur metabolism of the ruminant. Block *et al.* (1951) showed that, when sulphate containing ^{35}S was fed to a ewe, 4 hr. after dosing the bulk of the isotope was in the trichloroacetic acid-insoluble fraction of the rumen contents. This suggests a rapid incorporation of sulphate S into the rumen protein and in view of the present results, hydrogen sulphide may be an intermediate in this process. Several workers have shown that sulphide can be utilized by animal tissues in the production of sulphur-containing amino acids (Dziewatkowski, 1946; Melchior & Tarver, 1947; Smythe, 1943). It has also been shown in the goat that labelled sulphur, fed as sulphate, rapidly enters the milk proteins; the labelling was found equally in the cystine and methionine (Block *et al.* 1951). Some radioactivity was also found in the trichloroacetic acid filtrate of the milk. Thus it was suggested that some sulphate was absorbed from the rumen, some converted into protein of rumen contents and also part finally found in the amino acids of milk proteins. The significance of the present work may lie in the demonstration of the formation of sulphide from sulphate, which may then be utilized in the rumen for the production of the sulphur-containing amino acids of the protein of the bacteria.

SUMMARY

1. Sulphate is reduced to sulphide in the rumen of the sheep and also *in vitro* using a washed suspension of rumen contents under various conditions.

2. It was shown *in vitro* using hydrogen gas as hydrogen donor, that the optimum pH of the reduction is around 6·5.

3. Sulphite and thiosulphate are also reduced to sulphide.

4. In addition to hydrogen gas, the following hydrogen donors were also effective: glucose, formate, fructose, lactate, pyruvate, succinate, ethanol, citrate and malate.

The author is indebted to Dr S. R. Elsden for his constant help and advice and to Drs S. R. Elsden and I. W. McDonald for the preparation of the sheep fitted with permanent rumen fistulae.

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A Simple Technique for the Estimation of Radioactive Components of Plasma after the Administration of Radioactive Iodide

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Radioactive components of plasma released from the thyroid gland after the administration of ^{131}I have recently been studied in rats and in cases of thyroid disease by using in conjunction the methods of paper chromatography and radioautography (for a review, see Gross & Pitt-Rivers, 1952). The use of these techniques is time-consuming and not suitable for plasma containing small amounts of radioactivity. Furthermore, we have found losses of radioactive iodide to occur on paper chromatograms, whilst a similar observation has been made in the case of thyroxine by Taurog, Chaikoff & Tong (1950); the technique therefore is not suitable for quantitative work. This communication describes the use of methanol and silver phosphate for the fractionation of plasma components containing radioactive iodine. It has been found that silver phosphate will remove iodide and thyroxine from solution under specified conditions, whilst methanol liberates thyroxine from its state of physiological combination with plasma proteins; at the same time any thyroid protein present is precipitated with the proteins. Suitable application of these two reagents enables comparative estimations of the above components to be made with rapidity and accuracy. The determinations may easily be carried out on small serial samples of plasma, and enable graphs to be constructed of the relative changes occurring in the various fractions after doses of radioactive iodide have been delivered to the gland. The results obtained in several patients and in rats are described and correlated with chromatographic and radioautographic studies.

EXPERIMENTAL

Precipitation of plasma proteins. (*a*) Methanol. Plasma (0·5 ml.) was mixed thoroughly with methanol (1·16 ml.) and centrifuged.

(*b*) $(NH_4)_2SO_4$. Plasma (0·25 ml.) was diluted with 27·8% (w/v) $(NH_4)_2SO_4$ soln. (4·75 ml.) and a sample (0·2 ml.) taken immediately for counting. The suspension of globulins was then removed by centrifugation and a similar sample of the supernatant taken for counting. This procedure was adopted in order to eliminate the absorption error in counting, which error was due to the high salt content.

Treatment with Ag_3PO_4. (*a*) Plasma (0·5 ml.) was diluted with 0·067 M phosphate buffer, pH 7·4 (2·0 ml.) and the solution vigorously shaken for at least 5 min. with Ag_3PO_4 (about 100 mg.).

(*b*) After removal of plasma proteins with methanol, the supernatant was diluted with an equal volume of phosphate buffer (pH 7·4) and then shaken with Ag_3PO_4. In each case the excess of Ag_3PO_4 was removed by centrifugation and the residual activity in the supernatant estimated. Since a plasma-protein film absorbs radioactivity, a correction factor for the methanol supernatant counts is necessary. Unless otherwise stated, samples for radioactive assay (0·1 ml.) were air-dried on thin aluminium trays and the radioactivity was estimated with a mica end-window counter. It was found advisable to add carrier iodide and thyroxine (about 100 μg./ml. of plasma) before fractionation procedures were carried out.

Chromatography. Samples (6 μl.) of whole plasma were chromatographed on filter paper (Whatman no. 1), using as developing solvent the upper layer of a mixture of either butanol:2N acetic acid (1:1) or butanol:dioxan:2N-NH_3 (4:1:5, v/v). One-dimensional ascending chromatography was used, and markers of L-thyroxine, 3:5:3′-tri-iodo-L-thyronine, 3:5-di-iodo-L-tyrosine and radioactive iodide were run with each plasma as required. After overnight development, the papers were dried at room temperature, dipped into ninhydrin solution (0·2% in acetone) and hung in a dark cupboard for 2 hr. to allow development of the coloured spots. The paper strips were then radioautographed, using X-ray 'no screen' film, and the positions of the radioactive spots compared with those of the marker substances.

Animal techniques. Inbred Wistar rats of an American strain were used. They were maintained on a paste diet (bread meal, 2 parts; whole-milk powder, 1 part) for 14 days before use. The daily intake of iodine was about 10 μg./150 g. body weight. About 40% of an intraperitoneal dose of carrier-free ^{131}I became localized in the thyroid gland in these experiments. At suitable time intervals after the dose, groups of five animals were killed with ether and exsanguinated via the heart. The blood was diluted with one-quarter volume of isotonic citrate and plasma obtained by sedimentation in the refrigerator. A mean sample from each group was examined by the techniques described above.

Preparation of thyroid extract. The thyroid glands were removed from five adult rats, 6 hr. after the intraperitoneal administration of carrier-free radioactive iodide (150 μc/200 g. body weight). They were ground with powdered glass in a mortar, ice-cold water being added in portions (5 ml. in all). Fresh, cold rat plasma (5 ml.) was then added, the solid removed by centrifuging and the radioactive extract frozen overnight. After thawing, a small amount of insoluble material was removed by centrifugation before use.

RESULTS

The example shown in Table 1 illustrates the application of methanol precipitation and silver phosphate treatment to plasma and thyroid extract from rats, 6 hr. after a dose of carrier-free radioactive iodide (75 μc/150 g. body weight). In this instance the addition of carrier iodide had no effect on the results. The residual radioactivity in plasma (4%) after direct treatment with silver phosphate is likely to be in protein-bound thyroxine, since its removal was unaffected by adding carrier iodide. The combination of thyroxine and plasma protein is broken by methanol so that all the radioactivity then remains in the methanol solution (Table 1). The saline thyroid extract was diluted with normal

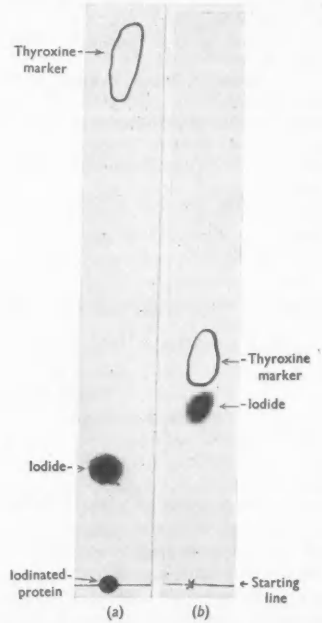

Fig. 1. Direct chromatographic and radioautographic analysis of rat plasma after administration of radioactive iodide, showing protein iodination in (*a*) when a mixture of butanol and acetic acid was used for development. The iodinated protein appears at the initial spot as can be seen by comparison with chromatogram (*b*), when butanol/dioxan/ammonia was used for development. The outlined areas indicate the positions of added marker substances developed with ninhydrin.

Table 1. *Application of methanol precipitation and silver phosphate treatment to rat plasma and thyroid extract, 6 hr. after administration of radioactive iodide* (75 μc/150 *g. body weight*)

	Percentage activity present as			
	Iodide	'Thyroid protein'	Thyroxine	Globulin
Plasma	96	0	4	0
Thyroid extract in normal rat plasma	3	99	0	98

rat plasma so that the thyroid protein could be precipitated along with the plasma proteins. Virtually all the thyroid activity was protein bound and associated with the globulin fraction of the added plasma. Chromatographic analysis of the thyroid extract confirmed that only a trace of radioactivity was represented by free iodide and thyroxine. We have established that iodide and free thyroxine are quantitatively removed by the silver phosphate treatment described. Free thyroxine has not been detected in any of the plasmas we have examined, so that the amount of radioactivity removed by silver phosphate is a measure of the percentage of iodide present. The accuracy of this measurement could only be diminished by the discovery of relatively significant amounts of free organic iodo-compounds in plasma, capable of reacting in a similar manner with silver phosphate. A similar process of reasoning applies to the treatment of the methanol supernatant from plasma with silver phosphate, which removes iodide and thyroxine, so that the percentage of the latter is given by difference.

We have encountered an iodination process occurring in plasma proteins when butanol/acetic acid was used for developing chromatograms in the presence of radioactive iodide (Fig. 1). This observation emphasizes the need for care in the interpretation of results when acid solvent mixtures are used. Acland (1952) has also commented on the liability to iodination of other plasma components when manipulations are carried out under acid conditions.

Application of methods to patients receiving therapeutic doses of ^{131}I. The results of the application of these techniques to patients at various intervals after the administration of radioactive iodide are shown in Figs. 2A and 2B. Case 1 (iodide-concentrating, secondary carcinoma thyroid; previous surgical thyroidectomy), who received three large doses of radioactive iodide (150, 200 and 380 mc) showed on each occasion an overall picture similar to case 2 (hyperthyroid, cardiac failure; dose, 8 mc). Soon after administration of these doses over 90% of the radioactive material in the plasma of case 1 was protein bound (i.e. not removed by direct

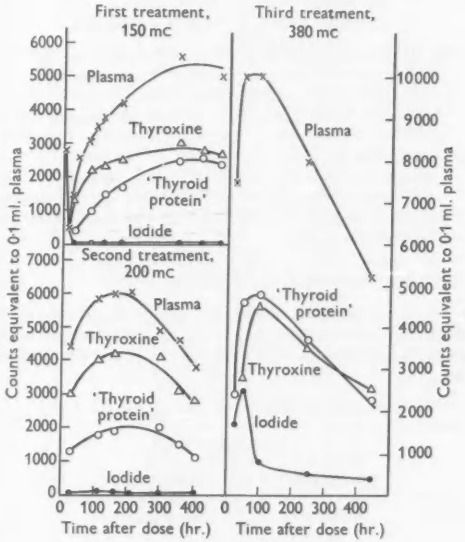

Fig. 2A.

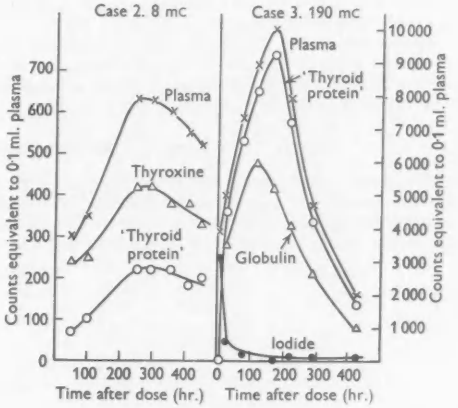

Fig. 2B.

Fig. 2A. Turnover studies of iodide, thyroxine and 'thyroid protein' in the plasma of a patient with an iodine-concentrating metastatic thyroid carcinoma, after surgical thyroidectomy. The patient received three treatment doses of radioactive iodide at intervals of several months. The estimations were made by means of the Ag_3PO_4 extraction and methanol-precipitation techniques described in the text.

Fig. 2B. Turnover studies of iodide, thyroxine and 'thyroid protein' in the plasma of two patients receiving therapeutic doses of radioactive iodide. Case 2 (hyperthyroid) received 8 mc, whilst case 3 (metastasizing thyroid carcinoma) received 190 mc of radioactive iodide to destroy the thyroid gland and possibly also the iodine-concentrating thyroid tumour. No thyroxine was detected in this latter case and practically the entire plasma radioactivity was associated with the 'thyroid protein' fraction.

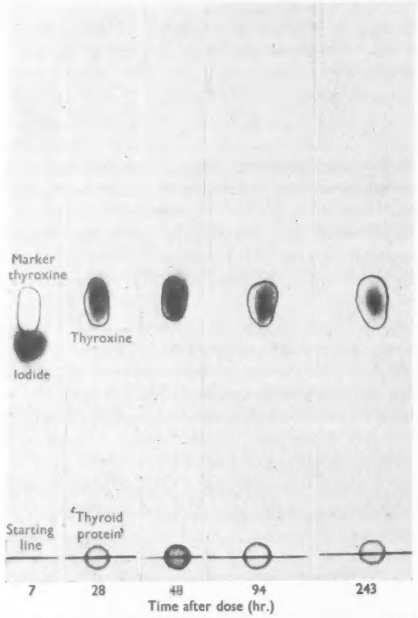

Fig. 3. Chromatographic and radioautographic analysis of plasma from case 1 (third treatment) reveals the presence of iodide only after 7 hr. In subsequent samples thyroxine was the only detectable soluble component. The tumour was discharging 'thyroid protein' into the plasma in the second sample (28 hr.), which persisted throughout the investigation. The outlined areas indicate the positions of added marker substances developed with ninhydrin.

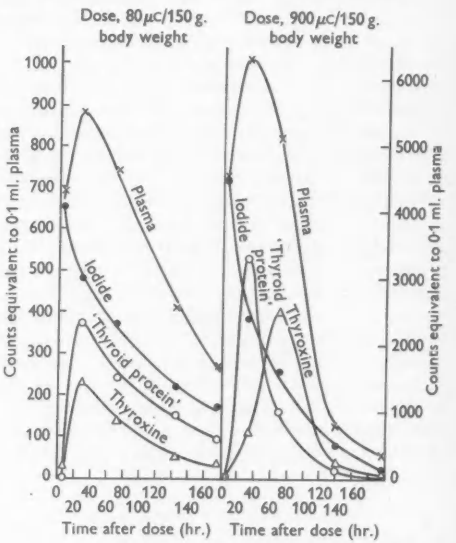

Fig. 4. Turnover studies of iodide, thyroxine and 'thyroid protein' in the plasma of rats after administration of radioactive iodide. After a dose of 80 μc the thyroid gland was only partially damaged so that the plasma curve includes some radioactivity representing thyroxine production by intact thyroid tissue. The dose of 900 μc caused total destruction of the gland, so that the plasma-radioactivity curve probably represents the metabolism of 'thyroid protein' absorbed into the blood from the disintegrated tissue.

Table 2. *Fractionation of plasma samples after injecting rats with radioactive iodide*

(The percentage changes in the radioactivity of rat plasma after treatment with Ag_3PO_4 and methanol, subsequent to the administration of a dose of carrier-free ^{131}I. The amount of 'thyroid protein' appearing in the plasma is related to the degree of damage to the thyroid gland. No measurable amount of 'thyroid protein' appeared in group 1, and the greatest quantity occurred in group 3 when the gland was totally destroyed.)

Group (dose)	Hr. after dose	Residual activity of plasma (percentage of total) after: Silver phosphate (loss = iodide)	Methanol precipitation (loss = 'thyroid protein')	Methanol followed by silver phosphate (loss = iodide + thyroxine)
1	6	0	100	5
(20 μc)	54	25	94	5
	121	57	100	—
	167	53	92	—
2	6	4	100	3
(80 μc)	27	47	77	5
	70	53	89	2
	142	48	83	2
	216	67	70	2
3	6	2	100	3
(900 μc)	27	61	48	5
	70	74	79	3
	142	38	80	6
	219	11	60	4

treatment of the plasma with silver phosphate). A considerable percentage of radioactivity remained unprecipitated by methanol and was then removed by silver phosphate, whilst an increasing amount remained associated with the methanol denatured proteins (see below). Typical results of the chromatographic and radioautographic analysis of plasma samples from case 1 (third treatment) are shown in Fig. 3, and show correlation with the analyses described above.

Case 3 (secondary adenocarcinoma thyroid; previous partial surgical thyroidectomy), received radioactive iodide (190 mc) to ablate functioning thyroid and possibly tumour tissue. This subject formed a complete contrast to cases 1 and 2, for with the exception of the first plasma sample which contained 80% of iodide, over 90% of the plasma activity was precipitated by methanol. Separation of the globulins with ammonium sulphate suggested that the iodine-containing complex was not homogeneous, for the radioactivity was not confined to this protein fraction (Fig. 2B, case 3).

Application to rats treated with radioactive iodide. Three groups of normal rats were given doses of ^{131}I of 20, 80 and 900 μc/150 g. body weight. Fractionation of the plasma samples after various times gave results shown in Table 2 and Fig. 4. After the lowest dose, no methanol-insoluble radioactive material was found in any plasma sample. Administration of 80 and 900 μc was followed by the appearance of methanol-insoluble radioactive substance within 24 hr., and the amount was greater at the higher dose level. The rate of disappearance of radioactivity from the plasma was much more rapid after 900 μc (Fig. 4). Examples of combined chromatographic and radioautographic analysis of whole plasma from these animals are shown in Fig. 5, and form an interesting comparison with those from cases 1 and 3 (Figs. 3 and 6).

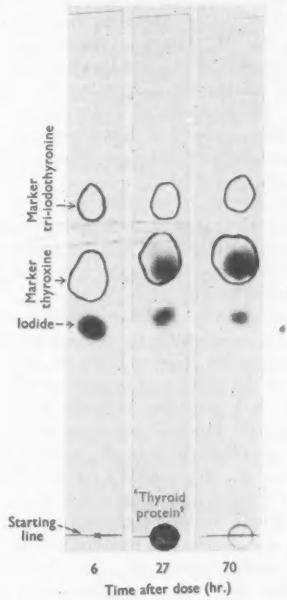

Fig. 5. Chromatographic analysis (in butanol/dioxan/ammonia) and radioautography of rat plasma 6, 27 and 70 hr. after administration of radioactive iodide (900 μc/150 g. body weight). Note the presence of 'thyroid protein' (lowest spot) and the areas corresponding to iodide and thyroxine. To be compared with Figs. 3 and 6. The outlined areas indicate the positions of added marker substances developed with ninhydrin.

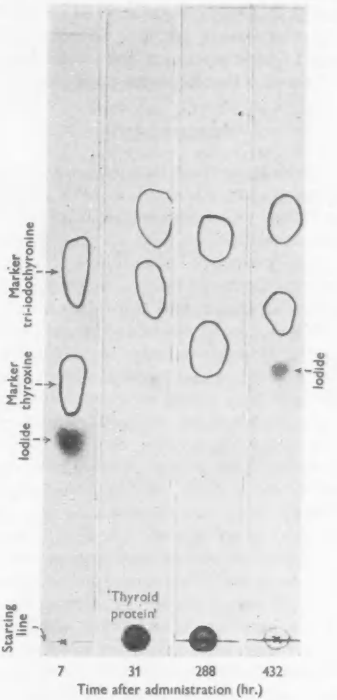

Fig. 6. Combined chromatographic and radioautographic analysis of plasma from case 3. Apart from the first plasma sample at 7 hr., the radioactivity was confined almost entirely to the 'thyroid protein' spot, the only other component detected being a little iodide. For comparison with Figs. 3 and 5. The outlined areas indicate the positions of added marker substances developed with ninhydrin.

Concurrent microscopical studies of the thyroid glands of rats from these experiments showed some damage when the dose reached 80 μc and complete disorganization after 24 hr. with 900 μc. A group of animals which had received the latter dose was retained for 4 weeks, when a further dose of the isotope (500 μc) was given. No radioactivity became localized in the thyroid region, and silver phosphate treatment of plasma samples after various time intervals showed that about 95 % of the circulating activity was iodide. The remainder was protein-bound material insoluble in methanol; this was confirmed by direct chromatographic and radioautographic analysis of these plasmas.

It is worthy of note that we have recognized the presence of di-iodotyrosine in the plasma of rats, 24 hr. after receiving 900 μc of ^{131}I. So far as we are aware, the presence of this substance in plasma has not previously been reported. Tong, Taurog & Chaikoff (1951) have demonstrated the presence of free di-iodotyrosine in the thyroid gland; its presence in plasma during the destruction of the thyroid gland is therefore not unexpected.

DISCUSSION

These results show that the application of methanol precipitation and silver phosphate treatment to plasma after the administration of ^{131}I readily furnishes quantitative data on the nature of the compounds present. Direct chromatographic analysis of the plasma samples in our series has shown that iodide and thyroxine were the only methanol-soluble components present in significant amounts. These two substances may be distinguished by direct treatment of plasma with silver phosphate in the first instance, which removes iodide; a similar operation on methanol-treated plasma estimates both thyroxine and iodide. Silver thyroxine must be highly insoluble under the conditions specified, for the amino acid is readily removed by silver phosphate even in the minute amounts present in plasma, and in the absence of added carrier thyroxine; it must, however, first be liberated from its protein-bound state by means of methanol. The efficiency of silver phosphate extraction of thyroxine has been confirmed using synthetic radioactive thyroxine added to plasma, when over 95 % was removed from methanol/phosphate buffer. Radioactive tri-iodothyronine behaves in similar fashion, combining firmly with plasma proteins when added to normal plasma. Like thyroxine, it is completely resistant to the action of silver phosphate until the proteins are denatured with methanol. The use of methanol and silver phosphate procedures does not distinguish between thyroxine and tri-iodothyronine but we have only encountered the latter amino acid in one plasma chromatogram where it formed not more than a few per cent of the total radioactive material present.

Of the three cases described (Figs. 2A, 2B), case 3 presented an entirely different picture from the others, in that throughout the series of samples (subsequent to the first) over 90 % of the radioactive iodine content of the plasma was precipitated by methanol. This material is related to radiation damage of functioning thyroid or tumour tissue (see below) and is now referred to as 'thyroid protein' for convenience. Its stability to methanol is in contrast to the physiological combination of thyroxine with plasma proteins, which is quantitatively broken when the proteins are denatured by the methanol. The soluble residue after methanol treatment in case 3 was iodide, no thyroxine being detected (Fig. 6). The unexpected absence of the latter substance, even from the earlier samples, may imply that the dose of ^{131}I given (190 mc) was sufficient to inhibit the function of the iodine-concentrating tissue present within a few hours, or that it was primarily incapable of producing thyroxine. Reference to Fig. 2 shows that 'thyroid protein' continued to accumulate in the plasma for about 1 week, after which its disposal occupied a similar time. In this case, therefore, we are apparently observing the results of rapid destruction of thyroid substance and/or tumour tissue, commencing within 24 hr. of ingestion of the therapeutic dose. This was apparently complete as judged by a subsequent dose of radioactive iodide (5 mc) some weeks later.

In case 2, the radioactive iodide administered (8 mc) caused transient damage as judged by the proportionate activity of the methanol precipitate (Fig. 2B); no chromatographic evidence is available from this treatment.

Although in case 1 the metastatic tumour concentrated a considerable proportion of the ^{131}I given on each occasion, the amount of active 'thyroid protein' in the plasma only reached about 50 % of the total radioactivity present (Fig. 2A). This maximum was reached very slowly in the first and second treatments in spite of the large doses given, in contrast to the rapidity with which the destructive effects were so manifested in case 3 (within 24 hr.). In the third treatment (380 mc) the peak amount of 'thyroid protein' in the plasma was attained much more rapidly (Fig. 2A), but this proportion was still little more than 50 % of the total radioactivity present. The failure to achieve a near-total conversion of plasma radioactivity into 'thyroid protein' as in case 3, in spite of the retention of a presumably adequate fraction of the large dose of ^{131}I administered, is reflected in the necessity for successive treatments. Another interesting feature in this case was the appearance of iodide in significant amounts after the third dose, whereas it

rapidly disappeared from the plasma in the previous treatments. The predominance of thyroxine in the plasma in its normal state of physiological combination with plasma proteins is not easily interpreted in view of the rapid uptake and destructive potentialities of the radioactive iodide administered. The application of these techniques to further cases is clearly indicated before solutions to these questions may be offered, but the diversity of the results presented here suggests the possible value of future investigations of this kind.

Experiments with rats were undertaken in an attempt to reproduce the results obtained in case 3, and to relate the extent of thyroid damage to the amount of 'thyroid protein' present in plasma. Tong, Taurog & Chaikoff (1952) have already noted the presence of this latter substance by direct chromatography of rat plasma after relatively large doses of radioactive iodide; we have confirmed their chromatographic observations (Fig. 5) and have also applied the methanol and silver phosphate techniques to the plasma samples (Fig. 4). Our data indicates that the onset of thyroid damage occurs more rapidly than was the experience of Tong *et al.* (1952), although the percentage uptake of the dose by the thyroids was the same in both series of experiments. A dose of 900 μc/150 g. body weight caused only the transient appearance of about 50 % of the plasma maximum radioactivity as 'thyroid protein' (Table 2), although the glands were completely destroyed. This contrasts with case 3, where over 90 % of the plasma radioactivity was precipitable by methanol throughout. In rats, the methanol-soluble material was composed of thyroxine and iodide (Fig. 5) compared with iodide only in case 3 (Fig. 6) and thyroxine only in case 1 (Fig. 3).

Reference to Fig. 4 shows that the total plasma radioactivity fell much more rapidly in rats receiving 900 μc than in those given 80 μc. In the former animals, the iodo-protein liberated from the disintegrating thyroids appeared to be quickly metabolized (probably forming the source of the thyroxine and iodide). The radioactive components of the plasma from rats receiving 80 μc will be represented in part by the production of thyroxine by the normal route from undamaged cells, and account for the slower turnover of active material in these animals. After the lowest dose (20 μc/150 g. body weight) there was no evidence for the formation of 'thyroid protein', from which it might be inferred that this quantity of radioactive iodide does not cause measurable damage to the gland (Table 2). So far as conclusions may be drawn from these preliminary studies, the metabolic pathway of the 'thyroid protein' in plasma appears to be different in man (case 3) and the rat (900 μc dose series). These are directly comparable since the radiation dose was sufficient to obliterate all iodine-concentrating tissue; thus the plasma picture reflects the absorption and metabolism of thyroid iodo-protein from the area of destruction. It may be that iodinated protein from tumour is included in case 3. Where functioning cells apparently continue to exist as in case 1, the metabolism of the iodo-protein is clouded by the continued formation of thyroxine. In rats, our failure to imitate the therapeutic results achieved in case 3 as regards the percentage of methanol-insoluble material in the plasma, is due to its rapid metabolism. Our observation that iodo-protein extracted from rat thyroids is wholly precipitated with the globulins of plasma (Table 1) suggests that the comparable material in the plasma of case 3 was not homogeneous, since it was only partially precipitated with the globulin fraction (Fig. 2B).

Damage to both thyroid tissue and iodine-concentrating tumour is clearly related to the appearance of 'thyroid protein' in the plasma. The question arises whether the quantitative assessment of this substance and thyroxine in the plasma after ^{131}I administration may be of value in the measurement of radiation damage and the effect of treatment. The construction of plasma fractionation curves as described, may enable some correlation to be made between dose and effect and throw light on the biochemistry of iodine fixation in tumour tissue.

SUMMARY

1. The use of silver phosphate treatment and methanol precipitation of plasma proteins is described for the rapid quantitative determination of the relative proportions of thyroxine and iodide in plasma following the administration of radioactive iodide.

2. Methanol precipitation enables the proportion of radioactivity associated with the plasma proteins which is not associated with thyroxine to be measured with facility.

3. The findings in three patients treated with radioactive iodide have been compared with the results of administering destructive doses of radioactive iodide to rats. In all instances the findings were corroborated by application of chromatography and radioautography.

4. The results so far suggest that the metabolism of protein material from the thyroid gland, which appears in plasma after destructive doses of radiation, follows different pathways in man and rat.

5. It is suggested that the construction of curves showing the variation in the relative amounts of these radioactive components of plasma in patients treated with radioactive iodide may prove of value in assessing the effects of treatment. Further light, too, may be thrown on the processes occurring in the thyroid gland and its pathological counterparts.

We wish to thank Dr R. Pitt-Rivers and Dr J. Elks for gifts of tri-iodothyronine and di-iodothyronine, respectively.

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The Resolution of Mixtures of C_{16}–C_{24} Normal-chain Fatty Acids by Reversed-phase Partition Chromatography

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Stand oils (fish drying oils) made from the body oil of the pilchard (*Sardina ocellata* Jenyns) are of commercial importance in South Africa and, as part of a general study of these and other polymerized oils (cf. Joubert & Sutton, 1952), the need arose for an analytical method suitable for the separation of mixtures of saturated and unsaturated acids having from 16 to 24 carbon atoms in the chain. For convenience, it was desirable that the method should be applicable on a semi-micro scale.

While the displacement chromatographic method of Holman & Hagdahl (1950) offered a possible approach, it was felt that the elaborate apparatus and technique involved would impair its ready applicability to our problem. For a similar reason an extension of the method of Vandenheuvel & Hayes (1952) seemed unlikely to be suitable, while that of Ramsey & Patterson (1948) was also rejected on the grounds that the higher fatty acids would have partition coefficients too greatly in favour of the less polar phase in the solvent system used.

The method of reversed-phase partition chromatography on benzene-rubber columns using methanol:acetone (3:1, v/v) containing varying amounts of water as mobile phase, has been applied by Boldingh (1950) to the quantitative semi-micro determination of saturated normal fatty acids from C_6 to C_{18}. Chromatography using rubber columns has been claimed by Boekenoogen (1952, not published in detail) to be satisfactory for resolution of mixtures containing saturated acids up to C_{24}, as well as for mixtures of various C_{18} unsaturated acids. Since 'Mealorub' rubber powder was unfortunately not available at the beginning of our work, the choice remained between elution chromatographic methods (Riemenschneider, Herb & Nichols, 1949; White & Brown, 1948; Herb, Witnauer & Riemenschneider, 1951), and the reversed-phase partition chromatographic method of Howard & Martin (1950). Recourse was had to the latter, which has been successfully extended to cover the resolution of mixtures of higher saturated fatty acids from 16 to 24 carbon atoms in chain length. Only acids with an even number of carbon atoms have been used for the investigation.

Preliminary experiments have indicated that the method should also be satisfactory for the resolution of unsaturated acids of differing chain length, as well as for acids of the same chain length but differing degrees of unsaturation (cf. Howard & Martin, 1950).

METHODS

The aqueous acetone-medicinal paraffin system of Howard & Martin (1950) was used throughout the investigation. Aqueous acetone concentrations are expressed on a v/v basis (e.g. 70% (v/v) acetone in water).

Preparation of materials

Non-wetting kieselguhr. 'Hyflo Super-cel' (Johns Manville, U.S.A.) was submitted to a process of flotation in water to remove the finer particles. About 5 lb. of kieselguhr were thoroughly mixed with 3 gal. of water and allowed to stand 1 hr., after which the suspended particles were decanted. The process was repeated on the sediment once, and the final product so obtained was dried at 110°. When cool, the material was exposed to the vapour of dimethyldichlorosilane in a partially evacuated desiccator. After 2 hr., the kieselguhr was washed free of acids with absolute methanol and dried at 110°. The product was completely non-wetting when shaken with water.

Medicinal paraffin. Colourless liquid paraffin (sp.gr. 0·880–0·895) was dissolved in pure pentane and percolated through a column of Brockmann grade I alkaline alumina to ensure removal of any acidic material. Evaporation of the pentane gave pure paraffin, used as stationary phase.

Acids. Palmitic, stearic and lignoceric acids were prepared from natural sources by distillation. Arachidic acid

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was synthesized from ethyl hydrogen adipate and palmitic acid, and behenic acid from ethyl hydrogen adipate and stearic acid using the electrolytic method of Greaves, Linstead, Shepheard, Thomas & Weedon (1950). All acids were purified by crystallization before use.

Preparation of columns

The mull used for packing the columns was prepared in batches. Non-wetting kieselguhr (93 g.) was suspended in a solution of paraffin (75 ml.) and anhydrous ether (500 ml.). The ether was slowly evaporated with constant agitation of the slurry, until a coarse homogeneous powder was obtained, which was then dried for 2 hr. at 60° in a vacuum oven.

In earlier experiments, a column 1·3 cm. in diameter and 30–35 cm. in height was found satisfactory for separation of component acids in mixtures, but it was later shown that resolution could be considerably improved by use of a column 85 cm. in height and 0·8 cm. in diameter.

Owing to difficulty in preparing air-free columns, special precautions were taken during packing. The mull (19·5 g.) was placed in a top-drive macerator, together with 83% aqueous acetone (200 ml.) previously equilibrated with paraffin. After thorough agitation, the slurry was transferred to a 500 ml. separating funnel constructed with a wide-bore stopcock and a ground glass neck into which a tap was fitted after introduction of the material. To expel all air, the slurry was boiled by holding the separating funnel in a horizontal position on a water bath. Next the column was filled completely with boiled, 83% aqueous acetone to give a positive meniscus at the top. The stem of the separating funnel was then closed with the tip of the forefinger and, by inclining the vessel, was filled with aqueous acetone. The funnel was attached to the column by judiciously sliding the stem over the liquid meniscus and withdrawing the finger at the same time. With the column and separating funnel stem completely full of liquid, the mull was allowed to fall into the column by setting it to flow. After some settling had taken place, the contents were compressed with air at an excess pressure of 50 mm. During subsequent settling, the column was vigorously tapped and shaken to ensure homogeneity of packing. A neatly fitting disk of filter paper was then placed on top of the packed mull. Satisfactory air-free columns were obtained by this method. All columns were jacketed and maintained at a constant temperature of 35°.

Loading of columns

The procedure employed by Howard & Martin (1950), of loading the acids dissolved in the developing solvent, was found unsuitable for our purpose owing to the low solubility of the longer-chain acids in aqueous acetone. Accordingly, the mixture to be chromatographed (containing 6–8 mg. of each component, accurately weighed) was dissolved in paraffin with gentle warming (0·2 ml. paraffin to each 7 mg. of acids). When cool, the solution was taken up in anhydrous ether (15 ml.). Non-wetting kieselguhr was then added (0·26 g. for each 0·2 ml. paraffin), and the ether was evaporated with continuous stirring. The powdery mull was then dried overnight under vacuum at 60°.

For loading, the mull was thoroughly slurried with 50% aqueous acetone (10–15 ml.) previously equilibrated with paraffin. The mixture was boiled to expel air and poured on to the column. After some settling, the solid mull was gently bedded by the slow downward motion of a plunger fitted with a perforated, stainless steel disk. After a filter-paper disk had been placed on top of the acid-containing band, the supernatant 50% aqueous acetone solution was run into the column. To prevent disturbance of the band and loss of acids during changes of developing solvents, a small protecting band of acid-free mull was placed on top of the column. For this purpose, a small quantity (about 1 g.) of mull was boiled with 50% aqueous acetone and packed with the plunger, as above, to a height of approx. 2 cm.

Developing solvents

Ordinary commercial acetone of low acidity was used to prepare developing solvents. Before use, all solvents were equilibrated with paraffin, and clarified by removing suspended oil droplets by percolation under pressure through a 3 cm. bed of kieselguhr-paraffin mull. The clear solutions were placed in separating funnels fitted with stopcocks and connected to a manifold of capillary tubing. To facilitate the changing of the developing solvents and also to provide a means of obtaining samples for blank titre determination, the column top was fitted as in Fig. 1.

While in use, the supply funnels were fitted with soda-lime guard tubes. The column eluate was also protected from contact with atmospheric CO_2 by means of a rubber collar and guard tube placed between the column tip and the siphon cup.

Siphon

A siphon was constructed to deliver 1·0 ml. samples. To prevent precipitation of higher acids in the siphon during cooler weather, it was wound with a 15 Ω length of nichrome wire and heated with a current of 0·35 A.

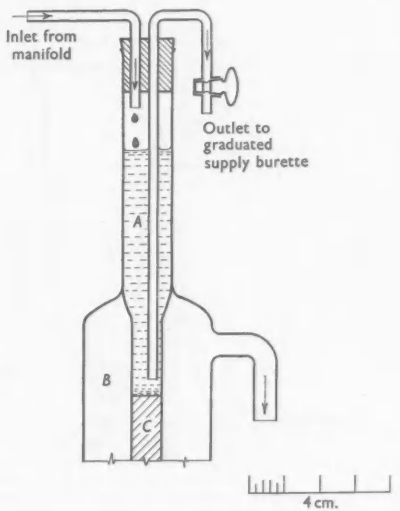

Fig. 1. Diagram of column head. *A*, developing solvent; *B*, column jacket; *C*, top of protecting band.

Titration of eluates

The titration vessel with N_2 circulation was used as described by Howard & Martin (1950). The $CHCl_3$ tube and light system were replaced by an ordinary 25 w globe built into a lamp-housing which surrounded the titration vessel, and was fitted with suitable filters to give a background of white light.

It was found necessary to saturate the N_2 stream with acetone vapour to counteract evaporation effects in the titration vessel. The N_2 stream was accordingly passed over Cu turnings at 700°, then through 40% aqueous KOH solution, and finally through a sintered-glass wash bottle containing neutral acetone, maintained at 30° by means of a small 1·5 w immersion heater.

Methanolic 0·01 N-KOH was protected at all points from contact with atmospheric CO_2. Bromothymol blue, as a 0·2% neutral solution in 70% aqueous acetone, was used as indicator.

Procedure

For chromatography of a mixture of C_{16}–C_{24} acids, development was begun with 70% aqueous acetone. This was continued until the C_{16} peak just began its downward slope, when a change was made to 75% aqueous acetone. The solvent was changed to 80% aqueous acetone on the downward slope of the C_{18} peak, to 83% aqueous acetone on the downward slope of the C_{20} peak, and finally to 90% aqueous acetone on the downward slope of the C_{22} peak. The hold-up was determined by running a narrow band of $KMnO_4$ solution down the column after the experiment. After each solvent change, the blank titre of the previous solvent was determined by titration of 10 ml. samples, under N_2, without boiling. Individual acids were chromatographed with solvent concentrations corresponding to those used for their elution from a mixture. A flow rate of 35 ml./hr. was used in all chromatograms.

RESULTS

A typical curve demonstrating resolution of a mixture of five acids is shown in Fig. 2. The areas underneath each peak were measured with a planimeter. The relative proportions of the areas gave the molar ratio of the component acids, while if each area was multiplied by the equivalent weight of the respective acid before proportionation, the weight ratio was obtained.

In an ideal case, where 100% recovery is achieved for all acids, the actual weight of each present can be determined if standardized alkali is used. In actual fact, recoveries were lower than the 95–100% reported by Howard & Martin (1950). In individual chromatograms, recoveries of C_{16}, C_{18}, C_{20} and C_{22} acids were, on the average, 25% low, while recovery of C_{24} acid was not greater than 60%. Efforts to improve recoveries by 'saturating' columns with higher homologues, before chromatography of individual acids, were unavailing. Some lost acid could be recovered and determined by stripping the column mull with ether, and titrating the acid:paraffin mixture in 50% benzene:neutral ethanol solution.

Attempts to eliminate the solvent blank titre determination, by using solvents neutralized either directly with alkali, or by percolation through beds of Amberlite I.R.A. 400, resulted in loss of chromatographic resolution.

The results of chromatographing eight artificial mixtures of C_{16}–C_{24} saturated acids are given in Table 1.

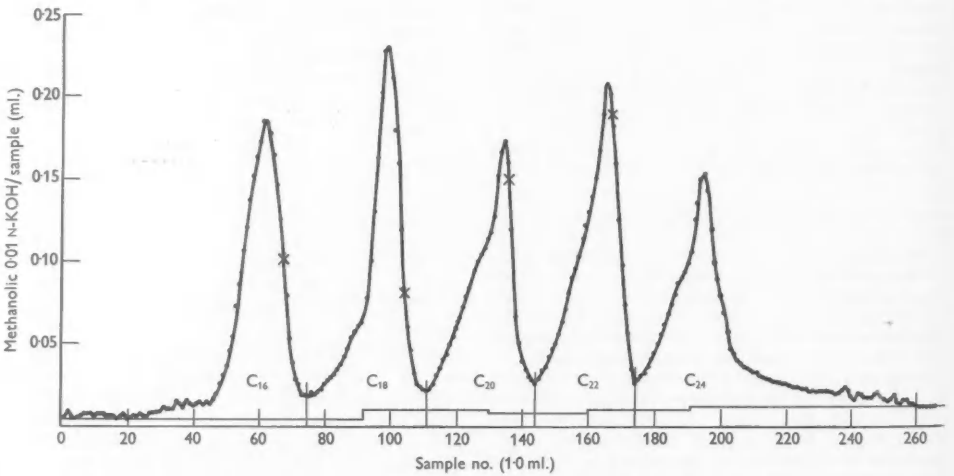

Fig. 2. Resolution of a mixture of palmitic, stearic, arachidic, behenic and lignoceric acids. Points marked ' × ' represent points of change to higher concentrations of aqueous-acetone developing solvent. The blank titre of the solvent in the eluate is represented by the lower line. Steps correspond to effective solvent-change points. Column 82 × 0·8 cm. diameter. Hold-up, 25 ml. (Note how effective solvent-change points correspond to steeper rises in the curve.)

Table 1. *Resolution of mixtures of C_{16}–C_{24} fatty acids*

(The weight percentage of each acid added in the mixture is shown in column *a*. The weight percentage experimentally determined is shown in column *b*. The percentage error is shown in column *c*.)

Expt. no.	Total wt. of acids (mg.)	Components														
		C_{16}			C_{18}			C_{20}			C_{22}			C_{24}		
		a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
1	32·20	26·2	24·4	−6·9	26·6	22·8	−14·3	24·1	26·8	+11·2	23·1	26·0	+12·5	—	—	—
2	40·70	20·0	18·4	−8·0	21·0	19·5	−7·1	20·0	21·6	+8·0	19·8	19·0	−4·0	19·2	21·4	+11·5
3	39·92	17·6	16·6	−5·7	20·4	18·5	−9·3	19·5	20·0	+2·6	21·7	21·8	+0·5	20·8	23·1	+11·0
4	39·23	18·4	16·4	−10·9	20·2	19·4	−4·0	19·4	19·5	+0·5	20·4	20·7	−3·3	20·6	24·0	+16·5
5	39·07	18·7	17·6	−5·9	20·1	19·7	−2·0	19·2	20·2	+5·2	21·2	20·9	−1·4	20·8	21·6	+3·8
6	31·42	23·0	23·9	+3·9	26·4	28·0	+6·1	25·4	23·5	−7·5	25·2	24·6	−2·4	—	—	—
7	66·59	18·9	18·4	−2·6	20·8	18·3	−12·0	19·9	19·2	−3·5	21·0	24·7	+17·6	19·4	19·4	0·0
8	33·82	17·9	16·4	−8·4	20·1	17·8	−11·4	18·9	17·1	−9·5	20·7	23·3	+12·5	22·3	25·4	+13·9
Av. error (%)		—	—	−5·6	—	—	−6·8	—	—	+0·9	—	—	+4·0	—	—	+9·5

DISCUSSION

Satisfactory qualitative resolution has been obtained for mixtures of acids from 16 to 24 carbon atoms in chain length. Where it is desired to determine only the weight ratio or molar ratio of the component acids in a mixture, there seems justification for assuming a quantitative accuracy of ± 10%, as evidenced by the results in Table 1. Low recoveries of acids do not permit of absolute weight determinations being made.

Adsorption effects on the non-wetting kieselguhr may possibly account for losses of acid, but attempts to find other supports were unsuccessful. Among others, cellulose powder, glass powder, and silica powder were investigated. Attempts were made to render these materials non-polar by exposure to the vapour of either dimethyldichlorosilane or trimethylchlorosilane, and by use of 'Quilon' (cf. Kritchevsky & Calvin, 1950). In no case was the treated material able to retain the paraffin phase of the solvent system.

The use of bromothymol blue as indicator may to some extent also contribute towards the observed low recoveries of acids. When added during a pH titration of 0·002N lignoceric acid with methanolic 0·01N potassium hydroxide in 90% aqueous acetone, the indicator showed a pK of 10·5, while the pH at the end point was found to be nearer 11·5. Thus bromothymol blue gives a low end point but all acids from C_{16} to C_{24} were found to titrate low by the same amount (10%) when titrated in a variety of aqueous acetone concentrations. Thus, equivalent quantities of acids will give equivalent peak areas, if no losses are incurred on the column, but these areas will all be low by the same amount. Quantitatively, acid will appear to have been lost, but the apparent loss will be the same for all peaks. Since the purpose of our investigation was to determine relative proportions of acids, rather than actual amounts, bromothymol blue was considered a satisfactory indicator.

The method does not appear suitable for the separation of corresponding normal-chain and *iso* acids. For example, it was not possible to resolve a mixture of stearic and 16-methylheptadecanoic acids. Application of the method to crude mixtures of unsaturated acids obtained by molecular distillation of pilchard-oil acids, has shown excellent qualitative resolution, when a temperature of 10° and aqueous acetone concentrations of approximately 60% are employed. Chromatography of such crude fractions in a saturated condition shows curves with fewer peaks, indicating that acids with the same chain length but differing degrees of unsaturation are resolvable. The application of the method to the total analysis of pilchard-oil acids, and its adaptation to a preparative scale, are being studied.

SUMMARY

1. The reversed-phase partition chromatographic method of Howard & Martin (1950) has been extended to cover the resolution of mixtures of even-numbered fatty acids from C_{16} to C_{24}, in fulfilment of a need for an analytical method capable of identifying acids above 18 carbon atoms in chain length.

2. The method has been shown to be of semi-quantitative as well as qualitative value, and is suitable for application to the analysis of natural mixtures of saturated and of unsaturated acids.

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The Metabolism of Short-Chain Fatty Acids in the Sheep

2. FURTHER STUDIES WITH RUMEN EPITHELIUM

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Short-chain fatty acids are produced in considerable quantities as a result of microbial activity in the fore-stomachs and large intestine of ruminants and are undoubtedly an important source of energy for these animals. In the first paper in this series (Pennington, 1952) it was shown that acetic, propionic and *n*-butyric acids can be utilized by many tissues of the sheep including the epithelia of the alimentary tract. Interest was focused principally upon the activity of the rumen epithelium, which proved to be a very useful tissue for *in vitro* metabolic studies. Some further experiments on the metabolism of short-chain fatty acids by this tissue are described in the present paper.

Particular attention has been given to propionic acid since little is known with certainty about the pathways of metabolism of this acid in animal tissues. It can be completely oxidized under suitable conditions by rat-liver preparations (Grafflin & Green, 1948; Huennekens, Mahler & Nordmann, 1951). The latter authors could demonstrate its oxidation by kidney preparations only when supplemented with a fraction from liver dispersions. Propionic acid is also known to be a precursor of carbohydrate in the dog (Ringer, 1912) and rat (Deuel, Butts, Hallman & Cutler, 1935–6). In this respect it may be of special importance to the ruminant, whose carbohydrate supply is limited owing to the nature of its diet and means of digestion. There is no generally accepted evidence that either acetic or butyric acid can give rise to a net increase of carbohydrate in animal tissues (Deuel, Johnston, Morehouse, Rollman & Winzler, 1945; Wood, 1946).

EXPERIMENTAL

The tissue was collected, prepared and incubated as described previously (Pennington, 1952) with the modification that the tissue sections, before incubation, were shaken vigorously for 5 min. in ice-cold Ringer solution in order to ensure a more complete removal of rumen micro-organisms possibly adhering to the tissue. In manometric experiments, where smaller amounts of tissue were needed, it was found convenient to use papillae, shaved from the rumen wall. These were pooled and usually 150 mg. (wet wt.) were introduced into each flask; since this quantity represents upwards of a dozen papillae, a uniform distribution of tissue between the flasks was ensured. Such preparations of papillae have a rate of respiration and fatty acid utilization somewhat higher than that of the tissue prepared in the usual manner. Chopping the tissue finely with scissors appreciably diminishes the rate of utilization of fatty acids.

Volatile fatty acids and ketone bodies were determined as before (Pennington, 1952). As previously mentioned, high blank values may be encountered in the determination of ketone bodies by the dinitrophenylhydrazine method of Greenberg & Lester (1944) unless the reagent is dissolved in a lower concentration of HCl (0·5 N, instead of 2 N recommended by these authors). Recrystallization of the reagent from butanol did not reduce the high blank values obtained when 2 N acid was used. Low blanks may also be obtained by dissolving the reagent in 2 N-HCl and extracting the solution twice with half its volume of CCl_4. The latter procedure is rather more convenient than the use of 0·5 N-HCl, owing to the difficulty in dissolving the reagent in acid of this concentration.

Glucose was determined by the method of Somogyi (1945) on $ZnSO_4/Ba(OH)_2$ filtrates. Glycogen was determined by the procedure of Good, Kramer & Somogyi (1933). Succinic acid was determined manometrically using a sheep-heart succinoxidase preparation (Umbreit, Burris & Stauffer, 1945).

Table 1. *Utilization of volatile fatty acids by rumen epithelium in media with and without calcium ion*

(Tissue (2 g.) incubated for 3 hr. at 39·5° with 100 μmoles of the Na salt of the fatty acids in the media indicated. The flasks containing the Ringer-phosphate media were gassed with O_2, and the Ringer-phosphate-bicarbonate with 80% O_2/20% CO_2. The figures are calculated to 100 mg. (dry wt.) of tissue.)

Expt. no.	Medium	Calcium	Fatty acid lost (μmoles)		
			Acetic	Propionic	Butyric
1	Ringer-phosphate-bicarbonate	+	6·6	12·5	23·2
		−	6·8	13·3	25·5
2	Ringer-phosphate-bicarbonate	+	.	27·5	.
		−	.	25·6	.
	Ringer-phosphate	+	.	2·8	.
		−	.	3·9	.

Table 2. *Utilization of propionic acid by sheep-rumen epithelium in presence of various concentrations of carbon dioxide*

(Tissue (2 g., wet wt.) incubated for 3 hr. at 39·5° in buffered Ringer containing 100 μmoles of sodium propionate. The values are calculated to 100 mg. (dry wt.) of tissue. The figures in parentheses are the percentages of CO_2 in the gas phase.)

Expt. no.	Propionic acid lost (μmoles)			
1	7·8 (0)	13·7 (5)	.	.
2	8·5 (0)	17·2 (5)	21·7 (10)	.
3	2·8 (0)*	6·5 (0)	.	.
4	12·2 (0)	15·4 (5)	17·6 (10)	18·3 (20)
5	13·6 (0)	23·4 (20)	22·7 (30)	23·0 (40)
6	4·5 (0)	29·4 (20)	.	.

* Flask contained KOH in a centre well to remove any CO_2 produced by endogenous respiration.

Standard manometric techniques were employed for the measurements of gas exchange by the tissue. In the measurement of O_2 uptake in 100% O_2 the conventional Warburg procedure, with 5% KOH in the centre well, was employed. The Dickens & Simer (1931) method was used for measurements in a 95% O_2/5% CO_2 atmosphere. Anaerobic glycolysis was measured under 95% N_2/5% CO_2, with yellow phosphorus in the centre well of the flasks to remove any traces of O_2. All Q values are expressed in μl. of gas/mg. dry wt./hr., the dry wt. being the final dry wt.

Media. The inorganic medium used for incubating the tissue was Krebs-Ringer solution (Umbreit *et al.* 1945), buffered with phosphate or bicarbonate, or both, as described in the text and legends. Since the calcium ion can exert certain inhibitory effects upon the metabolism of tissue preparations (see, for example, Krebs & Eggleston, 1940), it was first ascertained whether the presence of Ca^{2+} in this medium inhibits the utilization of fatty acids. Table 1 shows that this is not the case, and the complete medium containing Ca^{2+} was used in all the experiments.

All the values given in the tables are the means obtained from two runs with separate lots of tissues; analyses on each run were performed in duplicate. The tissues used in any one experiment were all obtained from the same animal. This was necessary since there was sometimes marked variation in activity between tissues from different sheep; duplicate tissue samples from the same animal usually gave results in close agreement.

RESULTS

Influence of carbon dioxide on fatty-acid metabolism. It was noted previously (Pennington, 1952) that the rate of disappearance of propionic acid incubated with rumen epithelial tissue of the sheep was greater under an atmosphere containing 5% of carbon dioxide than under oxygen alone, although there was little or no effect upon acetate or butyrate utilization. This was investigated further by measuring the disappearance of propionate with various levels of carbon dioxide in the atmosphere of the flask, the remaining gas being oxygen. The medium was Ringer-phosphate with the addition of the appropriate quantity of 0·154M sodium bicarbonate to produce the same initial pH in each flask. Table 2 shows the results obtained.

The effect of 5% of carbon dioxide in the gas phase upon the rate of disappearance of propionate was confirmed and the rate was further increased when the carbon dioxide level was raised above 5%. Since there was little change of pH during the metabolism of propionate this effect cannot have been due to the increased buffering concomitant with the increase in carbon dioxide tension. The uptake in flasks gassed with oxygen alone may be largely or wholly dependent upon the presence of carbon dioxide produced from endogenous substrates, since in one experiment (expt. 3) in which the centre well in a flask contained potassium hydroxide the uptake was further diminished.

It has not been found possible to demonstrate any effect of carbon dioxide upon the rate of metabolism of propionate by sheep-liver or -kidney slices. Possibly propionate is metabolized by a different

Table 3. *Utilization of acetic and butyric acids by sheep-rumen epithelium in presence and absence of carbon dioxide*

(Tissue (2 g.) incubated for 3 hr. at 39·5° in buffered Ringer containing 100 μmoles of fatty acid as Na salt. The figures are calculated to 100 mg. (dry wt.) of tissue.)

CO_2 in gas phase %	Fatty acid lost (μmoles)	
	Acetic	Butyric
0	7·1	32·5
20	6·9	33·0

Table 4. *Utilization of propionic acid by rumen epithelium in presence of various concentrations of fumarate*

(Tissue (2 g.) incubated for 3 hr. at 39·5° with 100 μmoles of sodium propionate and various concentrations of sodium fumarate in the media indicated. The flasks with Ringer-phosphate were gassed with O_2 and Ringer-phosphate-bicarbonate with 80% O_2/20% CO_2. The figures are calculated to 100 mg. (dry wt.) of tissue.)

Expt. no.	Medium	Fumarate concn. (M)	Propionic acid lost (μmoles)
1	Ringer-phosphate-bicarbonate	.	21·8
		0·010	21·2
		0·025	22·0
		0·050	20·4
2	Ringer-phosphate	.	1·0
		0·002	1·7
		0·010	2·6
		0·025	4·2
		0·050	4·5
	Ringer-phosphate-bicarbonate	.	14·7
3	Ringer-phosphate	.	1·5
		0·025	4·8
	Ringer-phosphate-bicarbonate	.	9·0
		0·025	8·8

pathway in these tissues, or it may be merely that a higher rate of production of carbon dioxide from endogenous substrates (or a different distribution of enzymes in the cells) may have made unlikely any effect of adding carbon dioxide.

Table 3 shows that the presence of 20% of carbon dioxide in the gas phase had no effect upon the utilization of acetate or butyrate.

In view of existing knowledge of the metabolic functions of carbon dioxide, the observed stimulation of propionate metabolism could be related to the supply of acids which constitute the Krebs's tricarboxylic acid cycle. Accordingly, the influence of fumaric acid upon the rate of disappearance of propionic acid was determined (Table 4). Fumarate, although increasing propionate uptake, was considerably less effective than carbon dioxide.

Effect of malonate. Malonate has been shown to inhibit the oxidation of fatty acids by liver tissue (Jowett & Quastel, 1935; Weinhouse, Millington & Friedman, 1949). Geyer & Cunningham (1950) reported an increase in the formation of ketone bodies by liver tissue from fatty acids in the presence of malonate; Weinhouse *et al.* (1949), however, found that malonate decreased the formation of ketone bodies. Jowett & Quastel (1935) found that malonate increased the endogenous production of acetoacetic acid. Table 5 shows the effects of malonate upon fatty-acid utilization and production of ketone bodies by rumen epithelium. It may be seen that the utilization of each of the acids was inhibited by malonate. Formation of ketone bodies from butyrate was depressed but there was little difference in the proportion of the butyrate converted into ketone bodies in the presence of the inhibitor. Malonate (0·025 M) did not influence the endogenous ketone-body production of the tissue, nor lead to the production of any ketones from propionate.

Table 6 shows the effect of adding fumarate upon the inhibition of propionate utilization by malonate.

Table 5. *Metabolism of volatile fatty acids by rumen epithelium in presence of malonate*

(Tissue (2 g.) incubated for 3 hr. at 39·5° in Ringer-phosphate-bicarbonate containing 100 μmoles of fatty acid as Na salt and the concentration of sodium malonate shown. Gas phase, 80% O_2/20% CO_2. Figures in parentheses are μmoles of ketone bodies produced. The figures are calculated to 100 mg. (dry wt.) of tissue.)

Expt. no.	Malonate concn. (M)	Fatty acid lost (μmoles)		
		Acetic	Propionic	Butyric
1	.	.	18·8	.
	0·008	.	15·5	.
	0·025	.	6·7	.
	0·081	.	0·5	.
2	.	5·4	18·2 (0)	24·5 (17·1)
	0·025	1·5	8·5 (0)	16·9 (12·6)

Table 6. *Utilization of propionic acid by rumen epithelium in presence of various concentrations of malonate and fumarate*

(Tissue (2 g.) incubated for 3 hr. at 39·5° in Ringer-phosphate-bicarbonate containing 100 μmoles of sodium propionate and other substances in the concentrations shown. Gas phase, 80% O_2/20% CO_2. The figures are calculated to 100 mg. (dry wt.) of tissue.)

Expt. no.	Concn. of malonate (M)	Concn. of fumarate (M)	Propionic acid lost (μmoles)
1	.	.	26·5
	0·025	.	17·1
	0·025	0·025	16·2
	0·025	0·050	16·9
2	.	.	22·4
	0·009	.	17·1
	0·018	.	11·7
	0·009	0·050	18·2
	0·018	0·050	16·8

The inhibitory effect of malonate suggested that oxidation of succinate may be an essential step in the metabolism of propionate. It was, therefore, of interest to determine whether, in the presence of malonate to inhibit succinate disappearance, the production of succinate from propionate could be demonstrated. The data given in Table 7 show that an appreciable part of the propionate disappearing under these conditions can be accounted for as succinate. The succinate figures would include any α-oxoglutarate which was present, since the ether extraction of the succinate was preceded by permanganate treatment to destroy malonate.

Effect of the ammonium ion. Edson (1935) showed that production of acetoacetic acid by liver slices is increased in the presence of ammonium chloride. In the course of experiments to determine whether ammonium chloride similarly influenced ketogenesis by rumen epithelium, it was observed that ammonium chloride partially inhibits the uptake of propionate by the tissue (Table 8). No such effect could be observed with butyrate, although the uptake of acetate, which is small with this tissue, appeared to be depressed. There was no effect on the production of ketone bodies.

The magnitude of the effect with propionate varied greatly from experiment to experiment. In some of the flasks, as indicated, the added ammonium chloride replaced an equivalent amount of sodium chloride in the medium; in the others the ammonium chloride was an addition to the medium; it was found, however, that the addition of equivalent amounts of sodium chloride did not depress the utilization of propionate.

Manometric studies. Some studies have been made of the respiration and glycolysis of the rumen epithelium. Fig. 1 comprises typical curves which were obtained showing the effect of fatty acids and of glucose upon the oxygen uptake of the tissue under 100% oxygen. Respiration data from several experiments are summarized in Table 9.

A totally unexpected result was the marked inhibition of respiration by propionate. Exactly similar results were obtained using propionate purified by steam distillation or by the use of 'purified propionic acid' (Hopkin and Williams Ltd.).

In view of the fact that carbon dioxide affects the rate at which propionic acid is metabolized (Table 1), the effect of propionate upon the respiration of the tissue was also examined in an atmosphere of 5% CO_2/95% O_2 (Table 10). Under these conditions quite a different result was obtained. Propionate markedly increased the Q_{O_2} of the tissue. It may be noted that the R.Q. of the tissue, which was low, was hardly altered when propionate was metabolized. There was an increase in bicarbonate, suggesting the conversion of propionate into non-acidic products.

Table 7. *Production of succinate by rumen epithelium*

(Tissue (4 g.) incubated for 3 hr. at 39·5° in Ringer-phosphate-bicarbonate containing the additions indicated. The percentage conversion is calculated after subtraction of the succinate production in the corresponding flask without propionate.)

Addition to medium	Propionate lost (μmoles)	Succinate* produced (μmoles)	Conversion (%)
None	.	2·8	.
Propionate (200 μmoles)	118	5·9	2·6
Malonate (0·009 M)	.	4·7	.
Propionate (200 μmoles) + malonate (0·009 M)	98	20·0	15·6

* Includes α-oxoglutarate: see text.

Table 8. *Metabolism of volatile fatty acids by rumen epithelium in presence of various concentrations of ammonium ion*

(Tissue (2 g.) incubated for 3 hr. at 39·5° in Ringer-phosphate-bicarbonate containing 100 μmoles of fatty acid as Na salt and the concentration of NH_4Cl shown. Gas phase, 80% O_2/20% CO_2. The ketone body values are given in parentheses and were derived by subtraction of the corresponding control figures. The figures are calculated to 100 mg. (dry wt.) of tissue.)

Expt. no.	Concn. of NH_4Cl (M)	Fatty acid lost and ketone bodies produced (μmoles)			
		Acetic	Propionic	Butyric	Control
1	—	5·8 (2·0)	27·6 (−1·1)	28·1 (24·9)	(2·2)
	0·015	5·0 (0·9)	22·6 (−1·2)	28·7 (26·4)	(2·3)
	0·030	4·5 (1·5)	18·2 (−1·2)	29·4 (26·9)	(2·4)
2	.	.	23·7	28·8 (24·8)	(2·0)
	0·030*	.	21·9	29·6	.
	0·060*	.	18·5	29·3	.
	0·090*	.	17·1	28·6 (23·8)	(2·0)
3	.	4·3 (1·6)	17·9	.	(2·3)
	0·030	2·8 (1·1)	4·5	.	(2·7)
	0·030*	.	7·2	.	.
	0·090*	.	3·1	.	.

* NH_4^+ replaced an equivalent amount of Na^+.

Table 9. *Rate of oxygen uptake of rumen epithelium in presence of fatty acids and glucose*

(Medium: 3 ml. of Ringer-phosphate containing 30 μmoles of substrate. Gas phase, 100% O_2. Temp. 39·5°. Values not marked by an asterisk were obtained using tissue sections as in preceding experiments. Where a pair of values are given, the first represents the initial rate, the second the rate after 3 hr. Where only one value is given the rate was constant for at least 3 hr.) (Q_{O_2} = μl. O_2/mg. dry wt./hr.)

	Q_{O_2}					
Expt. no.	No substrate	Glucose	Acetate	Butyrate	Propionate	Propionate + glucose
1	3·24	3·30	.	.	.	.
	2·41	.	.	.	.	.
2	3·28	3·85	3·04	5·67	2·93	.
	1·71	2·97	1·32	.	0·47	.
3	2·78	3·36	2·66	3·59	2·65	3·70
	.	.	.	.	0·66	.
4	3·69	.	.	6·75	3·75	.
	2·33	.	.	4·44	1·14	.
5	2·13	.	1·75	.	1·00	.
	0·97	.	0·60	.	0·25	.
	4·30*	.	.	.	.	.
	1·80*	.	.	.	.	.
6	2·61*	.	.	.	2·22*	.
	1·72*	.	.	.	0·59*	.
7	2·13*	.	.	.	1·18*	.
	0·11*	.	.	.	0·07*	.

* Using papillae only.

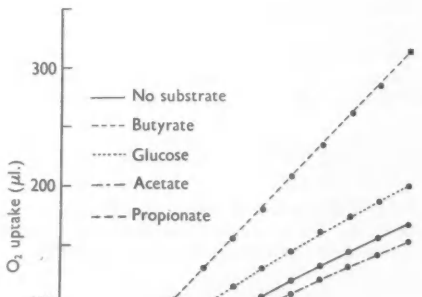

Fig. 1. Oxygen uptake of rumen epithelium in presence of fatty acids and glucose. Each flask contained 150 mg. (wet wt.) of tissue, 3 ml. Ringer-phosphate and 30 μmoles of the appropriate substrate. Fatty acids were added as Na salts. Gas phase, O_2.

Table 10. *Respiration of rumen epithelium in bicarbonate-buffered medium in presence and absence of propionate*

(Medium: 3 ml. of Ringer-bicarbonate containing 30 μmoles of sodium propionate, where added. Gas phase, 95% O_2/5% CO_2. Temp. 39·5°. The tissue used in expt. 2 was from the same rumen as that used in expt. 7, Table 9. (Q_{O_2} = μl. O_2/mg. dry wt./hr.))

Expt. no.	No substrate		Propionate	
	Q_{O_2}	R.Q.	Q_{O_2}	R.Q.
1	2·55	0·70	3·70	0·68
2	1·88	0·78	4·64	0·65

The rate of glycolysis of the tissue (carbon dioxide evolution with the bicarbonate medium under 95% N_2/5% CO_2) was found to be low ($Q^{N_2}_{CO_2} = 0{\cdot}64$). This was increased to 2·24 in the presence of glucose and was not measurably influenced by propionate, either in the presence or absence of glucose. This is in contrast with the results of Villasante & Ballowitz (1948), who reported that propionic acid completely inhibited the anaerobic glycolysis of liver slices.

Table 11. *Metabolism of volatile fatty acids by ox-rumen epithelium*

(Tissue (2 g.) incubated for 3 hr. in Ringer-phosphate containing 100 μmoles of fatty acid as Na salt. Gas phase, 100% O_2. The figures are calculated to 100 mg. (dry wt.) of tissue. The last three ketone body figures have been corrected by subtraction of the control value.)

Substrate	Change in volatile acid (μmoles)	Ketone bodies produced (μmoles)
None	+ 1·6	2·7
Acetate	− 5·8	1·5
Propionate	−10·3	−2·3
Butyrate	−22·3	16·8

As with other tissues, the presence of a relatively high endogenous respiration places a serious limitation on the usefulness of gas-exchange measurements. With some tissues, e.g. mammary-gland slices (Terner, 1951) it is possible to diminish this considerably by preliminary incubation in an inorganic medium. As may be seen from Table 9, the rate of oxygen uptake of rumen epithelium tends to fall off with time, although to greatly varying degrees. However, it was found that the increment due to added substrate (butyrate) was diminished when the substrate was added after a few hours.

Fatty-acid utilization by ox-rumen epithelium. The rumen of cattle is lined with an epithelium similar to that of the sheep. The data of Table 11 show that the uptake of volatile fatty acids by ox-rumen epithelium parallels that previously reported for the sheep.

DISCUSSION

Evidence for the occurrence of another site of carbon dioxide fixation has been obtained recently by Barban & Ajl (1951). They showed that the decarboxylation of succinate to propionate by *Propionibacterium pentosaceum* can be reversed. Although not necessarily a single-step transformation, it does not seem capable of an explanation involving any of the biological carboxylations previously reported, such as the conversion of pyruvate into oxaloacetate. The suggestion that animal tissues may convert propionate into dicarboxylic acids by a pathway not involving pyruvate has been put forward on the basis of isotope experiments (Lorber, Lifson, Sakami & Wood, 1950; Schreeve, 1952). Marshall & Friedberg (1952) have found that following injection of ^{14}C-labelled bicarbonate into rats the rate of appearance of radioactivity in the dicarboxylic acids of the liver was by far the most rapid in the cases of oxaloacetate and succinate.

The influence of carbon dioxide upon propionate metabolism in rumen epithelium shown above suggests that a carboxylation step is involved. The possibility that propionate is metabolized to pyruvate (Baldwin, 1952) and that carbon dioxide is acting to produce oxaloacetate from the latter must be considered; the oxaloacetate would be necessary for the oxidation of further pyruvate via Krebs's cycle. If this were so, it would be expected that the dicarboxylic acids of Krebs's cycle would be as effective as carbon dioxide in stimulating propionate metabolism. Similar reasoning was advanced by Ajl & Werkman (1948) to explain the ability of these acids to replace carbon dioxide for growth of *Escherichia coli*. The failure of fumarate to substitute completely for carbon dioxide (Table 4) may be taken as evidence against this possibility. It cannot have been due to an additional, competitive action of fumarate for enzymes or coenzymes involved in the metabolism of both substrates, since fumarate did not depress propionate uptake in the presence of carbon dioxide. Although fumarate did stimulate propionate uptake to some extent, this may have been due to carbon dioxide produced by oxidation of the fumarate. Respiration data (unpublished) indicate that fumarate is readily oxidized by this tissue. We have recently obtained further evidence (Pennington & Sutherland, unpublished results) that propionate is not necessarily metabolized via pyruvate in this tissue.

It is hardly possible as yet to draw any definite conclusion from the inhibition by malonate of the uptake of fatty acids by this tissue (Tables 5 and 6) in view of evidence (Pardee & Potter, 1949; Weinhouse *et al.* 1949) that the action of malonate is not confined to the inhibition of succinic dehydrogenase.

The failure to produce ketone bodies from propionate in the presence of malonate is a further fact which is difficult to reconcile with the conversion of propionate into pyruvate, since, in liver tissue, pyruvate is largely or wholly converted into acetoacetate in the presence of malonate (Lehninger, 1946; Recknagel & Potter, 1951).

The involvement of the tricarboxylic acid cycle in the metabolism of propionic acid could be put forward to explain the inhibitory effect of the ammonium ion upon the metabolism of the latter (Table 8). Recknagel & Potter (1951) investigated further the ketogenic effect of ammonium chloride reported by Edson (1935), and concluded that it was a result of the blocking of the tricarboxylic acid cycle owing to removal of α-oxoglutaric acid by reductive amination to glutamic acid. On the other hand, Crane & Ball (1951) postulated that ammonia also inhibits the fixation of carbon dioxide in animal tissues; this fits in with the above suggestion that propionate or one of its metabolic derivatives may undergo carboxylation. It may be noted that Huennekens *et al.* (1951) have reported an inhibitory effect of ammonia upon propionate metabolism by the liver 'cyclophorase system'. The

effect of ammonia upon the metabolism of the rumen epithelium may possibly be of physiological importance, since the concentration of ammonia in the sheep's rumen was found by McDonald (1948) to reach a level as high as 35 mg. ammonia N/100 ml. (0·025 M). This is higher than the lowest level found to exert an inhibitory effect *in vitro* (Table 8). McDonald also showed that ammonia is absorbed into the blood draining the rumen; levels of about 1·5 mg. ammonia N/100 ml. in the blood were found, although it appeared all to be removed by the liver.

The difference between the effect of propionate upon the oxygen uptake of the tissue in the presence and absence of carbon dioxide is very striking (Tables 9 and 10). In this instance the more commonly used Warburg procedure for measuring oxygen uptake gives totally misleading results. A tentative explanation of the difference may be suggested. The initial step in the metabolism of propionate may be combination with coenzyme A to form propionyl coenzyme A, the propionyl moiety being carboxylated in this form and further metabolized. In the absence of carbon dioxide, the coenzyme A may be immobilized as propionyl coenzyme A and thus rendered unavailable for the metabolism of endogenous substrates. The identities of the endogenous substrates responsible for the oxygen uptake are not known. The tissue does not contain measurable amounts of glycogen and the R.Q. is low (Table 10). It is of interest that Grafflin & Green (1948) found that propionate inhibits the oxidation of acetate by the kidney 'cyclophorase system', and Hill (1952) found that propionate inhibition of the growth of *Streptococcus faecalis* can be reversed by acetate.

It must be emphasized that this explanation of the effect of propionate upon respiration is entirely speculative, although Stadtman (1952) has produced evidence that propionate combines with coenzyme A in extracts of *Clostridium kluyveri*.

SUMMARY

1. The rate of metabolism of propionic acid by sheep-rumen epithelium increased with the proportion of carbon dioxide in the atmosphere up to about 20% CO_2/80% O_2. This level of carbon dioxide had no influence upon the rate of metabolism of acetic or butyric acid.

2. Malonate inhibited the uptake of each acid without showing any differential action on the formation of ketone bodies from butyrate. Malonate inhibition of propionate uptake was only partially reversed by fumarate. Succinate was produced when propionate was metabolized in the presence of malonate.

3. Ammonium ion inhibited the metabolism of propionate and possibly of acetate, but not of butyrate. It had no appreciable effect upon ketogenesis.

4. Propionate depressed the oxygen uptake of rumen epithelial tissue in the absence of carbon dioxide, but stimulated respiration in the presence of the latter. Butyric acid and glucose increased the rate of oxygen uptake.

5. The uptake of acetic, propionic and butyric acids, and production of ketone bodies by oxrumen epithelium paralleled that of the corresponding tissue of the sheep.

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The Enzymic Reaction of Amino Acids with Glutathione

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It has been shown that kidney extracts will bring about the exchange of L-amino acids for cysteinylglycine in reduced glutathione (GSH) to form the corresponding γ-glutamyl peptides (Hanes, Hird & Isherwood, 1950, 1952). The enzymic reaction of an amino acid, e.g. L-alanine with GSH, and the products formed in the system studied can be represented diagrammatically as follows:

γ-Glutamylcysteinylglycine + L-Alanine
↓ *γ-Glutamyl transferase*
γ-Glutamyl-L-Alanine + Cysteinylglycine
↓ *Peptidase*
Cysteine + Glycine

In the previous investigations the evidence for a reaction was the appearance of a new peptide on chromatograms of digest samples and was of a qualitative nature. A quantitative comparison of the reactivities of the various amino acids with GSH in the system under investigation involves the estimation of either a peptide or an amino acid, and the estimation must be made in the presence of other peptides and amino acids.

Several possible methods for the estimation of the reactivity of an amino acid with GSH were considered. (1) The estimation of the appropriate γ-glutamyl peptide formed. Large variations in the physical and chemical properties of these peptides would make each estimation an individual problem, especially since such estimations would have to be made in the presence of amino acids and closely related peptides. However, in some cases roughly quantitative estimations can be made by comparing the colour intensity of peptide spots after separation by paper chromatography.

(2) The estimation of residual GSH by use of glyoxalase. In the system being studied, two molecules of GSH would react to form the tetrapeptide γ-glutamylglutathione which might not act as a coenzyme for glyoxalase. If this were so, one molecule of cysteinylglycine would be formed but two molecules of GSH would 'disappear'. Thus there would be a disparity between residual GSH and the exchange reaction. This has indeed been shown by Waelsch (1952). Further, an amino acid reacting with γ-glutamylglutathione could set free a molecule of GSH and no cysteinylglycine.

(3) The estimation of cysteinylglycine and cysteine by the Lugg (1933) modification of the Sullivan method (1929). Initially this was thought to be suitable but it was found that during the estimation procedure GSH gave rise to significant amounts of cysteinylglycine and/or cysteine. The method was abandoned in favour of the following.

(4) The estimation of cysteine. The enzymic preparations contained an active peptidase which hydrolysed cysteinylglycine to cysteine and glycine. Preliminary experiments showed that the addition of certain amino acids to the enzyme-GSH system brings about the liberation of cysteine in excess of that liberated by the control without amino acid. The amount of cysteine produced in excess of that liberated by the control varies with the different amino acids and, with certain qualifications, can be taken as an index of their reactivity in the system.

EXPERIMENTAL

Estimation of cysteine

The method used is a modification of that developed by Nakamura & Binkley (1948). It depends on the blue colour produced specifically by cysteine in the presence of brucine and persulphate under acid conditions. Difficulties were encountered with the method. The colour intensity was found to be dependent not only on the concentration of cysteine but was also influenced by the concentrations of brucine, persulphate and hydrogen ions, as well as by time and temperature. However, these conditions can be standardized.

We found that tryptophan and methionine (cf. Nakamura & Binkley, 1948) seriously interfered with colour development and the method could not be used in their presence. In addition, ethionine was found to inhibit the colour production. The concentration of glycine was doubled to eliminate the small depression of colour production which was caused by amino acids. In the absence of cysteine the reagents produce a reddish colour, the absorption spectrum of which is reproduced in Fig. 1. This colour production falls off with increased cysteine concentration as shown. The absorption due to the red complex is negligible at 660 mμ., and optical densities have accordingly been measured at this wavelength. Absorption due to the red colour was not increased by addition of chloride which is known to react at a higher temperature to give a similar colour (Binkley, 1948). To enable low cysteine concentrations to be measured, this competing reaction, leading to the red complex, was prevented by adding 0·2 mg. cysteine to every estimation.

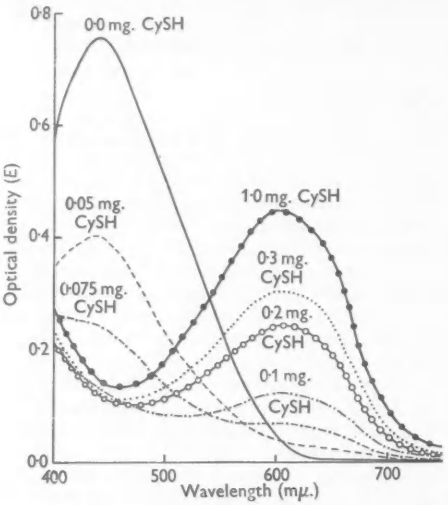

Fig. 1. Absorption curve of cysteine-brucine complex in the presence of varying amounts of cysteine. Recorded optical densities of solutions of cysteine-brucine complex were read against a water blank.

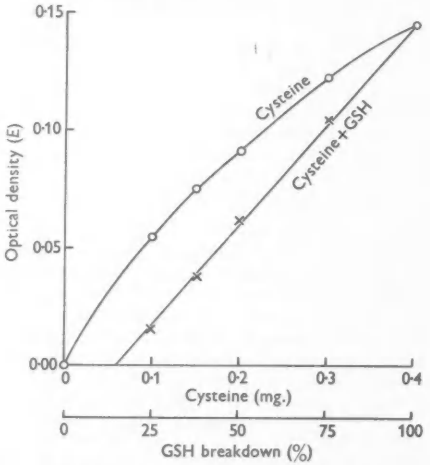

Fig. 2. Cysteine and cysteine-GSH calibration curves. Optical densities recorded were read against 0·2 mg. cysteine blank. In cysteine-GSH solutions the proportions of each were such as arise from GSH by hydrolysis. Optical densities were measured at 660 mμ.

GSH does not itself react with brucine to give a blue colour, but it does markedly reduce the colour intensity produced by cysteine (Fig. 2). To estimate cysteine in the presence of GSH, a separate calibration curve is therefore necessary. On the cysteine-GSH calibration curve each point represents cysteine and GSH concentrations corresponding to the amounts resulting from the progressive breakdown of GSH (1·012 mg.). This curve, in the range in which we are interested, is nearly linear, unlike the cysteine calibration curve. Because of the depression of colour production by GSH, the background cysteine enables smaller amounts of cysteine to be measured when GSH is present. Nakamura & Binkley (1948) do not record any interference by GSH. Their finding that in enzymic experiments GSH inhibits at higher concentrations has an alternative explanation in terms of the colour depression described.

Reagents

The reagents used were all of analytical quality.

Glycine-sulphuric acid. Glycine (5 g.) in 100 ml. of 12·5% (v/v) sulphuric acid. This reagent is stable for months.

Solution for background cysteine. Cysteine (0·2 mg., or equivalent amount of cysteine HCl) in 1 ml. water. This reagent is freshly made up daily.

Brucine solution. Brucine (1 g.) in 100 ml. 5% (v/v) sulphuric acid. This reagent is made up weekly.

Potassium persulphate solution. Potassium persulphate (0·5 g.) in 100 ml. water. This reagent is stable for at least 2 weeks.

Trichloroacetic acid (TCA). 50% (w/v).

Procedure

The colour was developed in test tubes which had a small process on the inside, just above the 8 ml. mark, to facilitate pipette drainage. The reagents were added in the following order: 2 ml. glycine/sulphuric acid reagent; 1 ml. cysteine reagent; 2 ml. brucine reagent; 2 ml. TCA filtrate containing from 0·0 to 0·4 mg. cysteine, or 2 ml. water in the control (the TCA and phosphate from the digest did not affect colour development); 1 ml. potassium persulphate reagent.

Optical densities were measured at 660 mμ. on a Beckman spectrophotometer, Model DU.

The reaction resulting in development of colour begins when the persulphate is added. The persulphate was therefore added to successive tubes at 1 min. intervals. After mixing of the contents, the tubes were immersed and the colour developed in a water bath at 30±0·1° for 45 min. The tubes were then removed and placed in an ice bath for 2 min. The blue solutions were transferred to the spectrophotometer cells and 4 min. after removal from the constant temperature bath the cysteine blank was read off against water (as a check on the reagents). For 0·2 mg. cysteine, $\log I_0/I$ at 660 mμ. was about 0·170. The background cysteine solution was then set at zero (5 min. after removal from bath) and the unknowns were read at minute intervals, all 45 min. after the persulphate additions. The amount of cysteine present or the GSH broken down was then read from the graph (Fig. 2). The method is reproducible to ±3%.

Estimation of cysteine produced from GSH by kidney extracts in the presence of amino acids

To enable the reactivities of a wide range of amino acids with GSH to be tested, it was convenient in most cases to use an enzyme concentration that brought about 35–40% GSH breakdown during the incubation period of 30 min. at 30°, i.e. in the absence of any added amino acid. Extracts

prepared in the following manner were suitable: 3 g. of frozen sheep kidney cortex were disintegrated in 20 ml. of ice-cold 0·2 M potassium phosphate buffer, pH 7·4, in a glass homogenizer for 30 sec. at 1400 rev. min.$^{-1}$. After centrifugation for 15 min. at 1500 *g* to remove the coarse fraction, the supernatant was stored at $-10°$ in 2 ml. portions until needed. Under these conditions the enzyme was stable for months, and it was therefore possible to compare a large range of amino acids using a single enzymic preparation. A small amount of cysteine and of GSH was present in the enzymic preparations. After incubation, cysteine (0·010–0·015 mg./2 ml. sample) was found in the six preparations tested. In any one enzymic preparation the endogenous cysteine was constant and therefore the order of reactivities of the amino acids was not altered.

The systems and samples for cysteine analysis were prepared as follows. To 2 ml. enzymic preparation in 0·2 M potassium phosphate buffer, pH 7·4, were added 2 ml. amino acid solution (neutralized when necessary) and 1 ml. GSH solution (6·32 mg./ml., 20·6 μmoles) in this order. This quantity of GSH corresponds on complete hydrolysis to 0·4 mg. cysteine in the final 2 ml. TCA supernatant. The GSH solution was prepared afresh before each incubation and dissolved in a vessel in which the air had been displaced by N_2. The reaction was carried out in stoppered test tubes under N_2 in a water bath at 30° for 30 min. At the end of this time, 4 ml. were transferred to a glass-stoppered 10 ml. centrifuge tube containing 5 ml. of water and 1 ml. of 50% TCA. The tube was stoppered, the contents were mixed, allowed to stand for 5 min. and then centrifuged lightly; 2 ml. of the supernatant was then used for analysis. Cysteine, when added to the enzymic preparations and incubated under the conditions used, could be recovered within experimental error, showing the absence of significant cysteine-desulphurase activity.

Chromatographic procedure

The system was on a scale of one-tenth of that used for cysteine estimations. To 0·2 ml. enzymic preparation in 0·02 M potassium phosphate buffer, pH 7·4, were added 2 μmoles neutralized GSH in 0·1 ml., and 0·2 ml. amino acid solution (neutralized when necessary). Incubation was carried out in stoppered 9 × 75 mm. test tubes in an atmosphere of N_2. The enzymic preparation was dialysed in a moving bag against 0·02 M potassium phosphate buffer for 4 hr. before use to remove amino acids present in the enzymic preparation. After 30 min. incubation at 30°, 1·0 ml. of warm ethanol containing 2·4 μmoles *N*-ethylmaleimide was added to precipitate proteins and block —SH groups (Hanes *et al.* 1950). Cysteinylglycine when coupled with *N*-ethylmaleimide in this manner is capable of being detected on chromatograms at a concentration of 0·001 M. Under the conditions used, this represents a figure of 5% GSH breakdown. After centrifugation, the supernatant was collected, the precipitate washed once with 0·2 ml. 66% ethanol (v/v) and the combined supernatant liquids evaporated *in vacuo* over NaOH in a small evaporating cup. The residue was then taken up in 0·05 ml. water and 0·002 ml. portions were used for chromatography. Solvents used were phenol saturated with water, butanol:water:glacial acetic acid (50:40:10, v/v), propanol:water (80:20, v/v) as such, and with 0·5 ml. NH_3 (sp.gr. 0·880) added to the chromatography jar. Time of development varied according to separation required: 15 hr. for short runs; up to 100 hr. for long runs. In the longer runs, papers had a thick pad of filter paper clipped to the bottom of the paper strip.

RESULTS

Trial experiments showed that with the concentration of GSH chosen, 20·6 μmoles/5 ml., and an amino acid concentration of 132 μmoles/5 ml., increases in cysteine liberated over a 30 min. incubation period could be observed. At this stage of the work the effect of the concentration of amino acids on the liberation of cysteine had not been determined. This aspect is discussed in the following section. Table 1 gives the excess of cysteine liberated from GSH by a wide range of amino acids and some of their derivatives. Estimations of the liberated cysteine were carried out in duplicate and always with a GSH control without added amino acid.

The results given in Table 1 show that L-α-amino acids differ markedly in their abilities to liberate cysteine from GSH. The DL-amino acids, where tested, gave approximately half the values of the

Table 1. *Comparative reactivity of various amino acids with GSH*

(System: as described in Experimental with 2 ml. enzyme preparation, 2 ml. amino acid solution containing 132 μmoles (control: 2 ml. water in place of amino acids) and 1 ml. solution containing 20·6 μmoles GSH. Incubation: 30 min. under N_2 at 30°. Figures represent additional cysteine liberated in excess of control by amino acids and are expressed in terms of % GSH breakdown. Figures in brackets calculated for L- acids from DL- acids × 2. Reactivities measured with a single preparation have been listed separately to enable more accurate comparisons to be made.)

Amino acid	GSH breakdown in excess of control (%): In a single preparation	GSH breakdown in excess of control (%): Results from other preparations
Glycine	33	26, 27, 28, 32
L-Alanine	24 (28)	17 (10)
DL-α-Amino-*iso*butyric acid	—	0
DL-α-Amino-*n*-butyric acid	12	6
L-Valine	—	2 (−1)
DL-Norvaline	—	22
L-Isoleucine	—	2 (0)
L-Leucine	21	16 (16)
DL-Norleucine	21	20
L-Phenylalanine	—	21, 25, 26
DL-Serine	20	17
DL-Threonine	3	3, 6
L-Aspartic acid	2	−1, −4
L-Asparagine	27	23
L-Glutamic acid	21	15, 16
L-Glutamine	37	42, 43, 44
L-Histidine	36	19, 21, 23, 24
L-Ornithine	17	—
L-Citrulline	37	—
L-Arginine	33	18
L-Lysine	22 (26)	20, 21, 24
L-Methionine sulphoxide	43	—

Table 2. *Comparative reactivity of the more reactive amino acids with GSH*

(System: as described in Experimental with 2 ml. enzymic preparation, 2 ml. amino acid solution containing 132 or 264 μmoles (control: 2 ml. water in place of amino acids) and 1 ml. solution containing 20·6 μmoles GSH. Incubation: for varying periods under N_2 at 30°. Figures represent additional cysteine liberated, in excess of control, by amino acids and are expressed in terms of % GSH breakdown. The controls corresponded to 12, 24 and 43% GSH breakdown for 5, 10 and 20 min. incubations respectively.)

Amino acid	Concentration (μmoles/5 ml.)	Time of incubation (min.) 5	10	20
		GSH breakdown in excess of control (%)		
L-Methionine sulphoxide	132	36	62	41
L-Glutamine	132	28	53	45
L-Citrulline	132	28	42	35
DL-Norleucine	264	11	26	—
DL-Norvaline	264	9	26	—

L-form; there is thus no pronounced inhibition by the D-form. Amino acids substituted on the amino group such as sarcosine, L-proline and L-hydroxyproline were not reactive. Tryptophan, methionine and ethionine interfered with the colour production by cysteine, and so quantitative data for these amino acids cannot be given. However, chromatographic evidence has been obtained to show that these amino acids react with GSH in the system. Tyrosine was too insoluble for comparisons with other amino acids to be made. Urea, ammonium chloride, 8-hydroxyquinoline and chloramphenicol had no effect on the system.

With the more reactive amino acids, over 80% of the GSH was broken down by the end of the reaction period. This resulted in a reduction of the amount of GSH available as a substrate and in progressive slowing of the reaction; thus, these amino acids are more reactive than indicated in Table 1. As the range of reactivities of the amino acids was too great for them to be compared on the same time scale, the most reactive ones were tested over shorter periods of time. The results are given in Table 2.

Effect of amino acid concentration on the liberation of cysteine

Figs. 3 and 4 show the effect on the liberation of cysteine from GSH of increasing concentrations of various amino acids. It can be seen that as the concentrations of L-aspartic acid, L-valine and L-isoleucine were increased, the liberation of cysteine decreased. In contrast, the other L-α-amino acids, up to a certain concentration depending on the amino acid concerned, caused an increase in the amount of cysteine liberated. At higher concentrations, however, there was a decline in the cysteine liberated, and in some cases it fell below the control with GSH alone, e.g. DL-threonine. The increase in cysteine liberation with increasing substrate concentration was probably due to increased saturation of the active centre of the enzyme. This increase in cysteine liberation was usually paralleled by an increase in corresponding γ-glutamyl peptide formation as judged from the chromatograms. Fig. 6 shows that at higher concentrations of glycine where cysteine liberation fell off, γ-glutamylglycine formation fell off also. At these high concentrations the residual GSH was high. This means that GSH was not being acted upon by the enzyme system. It is possible that the amino acids compete with GSH for the active centre of one enzyme, as seems to be the case with L-valine (Fig. 4). A decrease in cysteine liberation was also observed when the

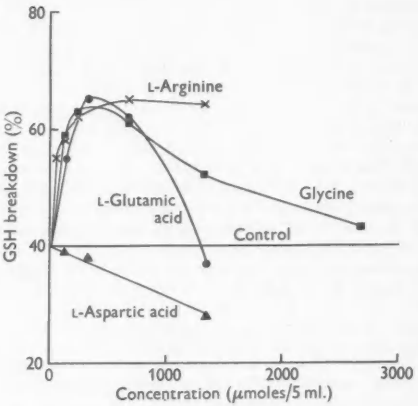

Fig. 3.

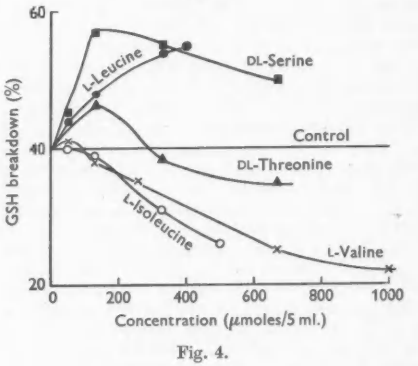

Fig. 4.

Figs. 3, 4. The effect of varying concentrations of amino acids on release of cysteine from GSH expressed in terms of % GSH breakdown. Conditions as in Table 1 but with varying concentrations of amino acids.

reacting amino acid was replaced by similar concentrations of sodium sulphate and glucose. The effect of high concentrations of amino acids could therefore be a non-specific one. As any point on the amino acid concentration curves must represent a combination of reaction and inhibition, comparison of the relative reactivities of amino acids in the system is only possible on the ascending slope of the concentration curve.

Paper chromatography as the index of transpeptidation

Hanes *et al.* (1950, 1952) have shown that L-glutamic acid, L-glutamine, L-phenylalanine, L-leucine, L-valine, L-tryptophan, L-cysteine and glycine would react with certain γ-L-glutamyl peptides, including GSH, to form new spots running in the expected positions for the γ-glutamyl peptide corresponding to the added amino acid. In many cases these spots were eluted, hydrolysed and re-chromatographed to establish their composition.

In the present work other amino acids have been tested for their reaction with GSH by the presence or absence of new spots on chromatograms after concentration of digest samples. Positive evidence has been obtained for the reactivity of L-arginine, L-asparagine, L-lysine, L-histidine, L-methionine, DL-ethionine, DL-norleucine, DL-norvaline, L-alanine, DL-serine and DL-threonine. γ-L-Glutamyltyrosine has been shown to serve as a donor peptide (Hanes *et al.* 1952), and it is probable that tyrosine will react with other γ-glutamyl peptides in the system. With the exception of L-isoleucine and L-valine the reactivity of amino acids as followed by the appearance of a new spot on a chromatogram was always paralleled by a positive figure for cysteine liberation by the amino acid concerned. Similarly, failure to detect a reaction by chromatography was paralleled by a zero figure for cysteine liberation in the case of L-aspartic acid, L-proline and L-hydroxyproline.

Further investigations on the transfer reaction

The positive results given by chromatography are not consistent with the negative results given by estimation of cysteine liberated from GSH by L-isoleucine and L-valine. The figures given for excess of cysteine liberated (Table 1) in these cases do not measure the relative reactivities of the amino acids with GSH. Accordingly, we have examined the enzymic reaction further in an attempt to determine the relationship between extra cysteine liberated by the amino acids and their relative reactivities.

The liberation of cysteine from GSH is a two-step process. First, cysteinylglycine is liberated as a result of a transfer reaction involving the γ-glutamyl linkage. Secondly, the cysteinylglycine so formed is hydrolysed to cysteine and glycine. The enzymic preparations, as well as catalysing the transfer of γ-glutamyl groups to amino acids, also form glutamic acid from GSH, presumably by hydrolysis. Fig. 6 shows that as the glycine concentration was increased up to a concentration of 67 μmoles/0·5 ml. digest, γ-glutamylglycine formation increased and glutamic acid formation decreased. This competition between water and glycine for the γ-glutamyl group in GSH makes it likely that the two effects, hydrolysis and aminolysis (transpeptidation) of the γ-glutamyl peptide bond, are properties of one enzyme. If this were so, the initial transfer reaction would involve water or the amino group of GSH as an acceptor. In the case of water as an acceptor, all three amino acids in GSH are liberated. In the case of an amino acid (including GSH) as an acceptor, cysteine and glycine only are liberated (the γ-glutamyl linkage being preserved in the new γ-glutamyl peptide formed). As the reaction proceeds there is a progressive increase in glutamic acid, cysteine and glycine and these amino acids can then participate in further transpeptidation reactions. The addition of other amino acids, with few exceptions, brings about the release of extra cysteine (due to extra γ-glutamyl peptide formation) at the expense of GSH. This is shown in the time/progress curve in Fig. 5 with glycine as the added amino acid. It is probable that each γ-glutamyl peptide formed serves as a donor peptide in further transpeptidation reactions.

For liberated cysteine to be taken as an index of transpeptidation activity, it is necessary that a relationship be established between cysteine liberation and γ-glutamyl peptide formation. Four aspects of the enzymic reactions that are concerned in such a relationship have been examined.

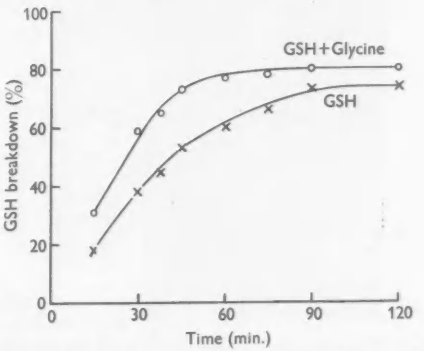

Fig. 5. Time/progress curve for % GSH breakdown with and without added glycine. System: 2 ml. enzyme preparation, 2 ml. glycine solution containing 660 μmoles, or 2 ml. water and 1 ml. solution containing 20·6 μmoles GSH. Incubation: varying intervals of time at 30° under N_2.

(1) *Hydrolysis of GSH.* If the two transfer reactions, hydrolysis and transpeptidation, are properties of a single enzyme, then there is likely to be competition between water and amino acids for activated γ-glutamyl linkages. Therefore, the higher the amino acid concentration the less should be the hydrolysis. The depression of hydrolysis by added glycine as gauged by glutamic acid formation is shown in Fig. 6. The cysteine produced in the control therefore includes a greater proportion of cysteine which has its origin in GSH hydrolysis than the cysteine produced from GSH in the presence of added amino acid. This means that amino acids are more reactive in transpeptidation than is shown by the figures for extra cysteine liberated (Table 1). It is likely, however, that the amino acids most reactive in transpeptidation are also the best competitors with water. For instance, Fig. 7 shows that glycine depresses glutamic acid formation more than does L-valine at the same concentration. It is also a stronger reagent in transpeptidation, as measured by γ-glutamyl peptide formation (Fig. 7) and by the liberation of extra cysteine (Table 1). As long as the two effects, transpeptidation and depression of hydrolysis, work proportionally and in the same direction with each amino acid, a comparison of reactivities can be made. The effect of amino acids on enzymic hydrolysis of GSH is at present being investigated further.

(2) *Hydrolysis of cysteinylglycine.* For cysteine to be an accurate measure of reactivity of amino acids with GSH, the cysteinylglycine formed must be hydrolysed completely and as rapidly as it is formed. The enzymic preparations used, however, did not carry the hydrolysis to completion. On concentrating the breakdown products ten times, it was possible to detect traces of cysteinylglycine on chromatograms. The addition of large amounts of cysteinylglycinase (prepared by the method of Binkley, 1952) to the digests did not result in either an increase in cysteine (measured spectrophotometrically) or a decrease in the cysteinylglycine present on chromatograms. It is possible, therefore, that the traces of this peptide which were present in the digests represented a final equilibrium mixture of cysteinylglycine, cysteine and glycine. Further, the trace amount of cysteinylglycine present in digests in the presence of various amino acids (132 μmoles/5 ml.) was constant as judged from chromatograms.

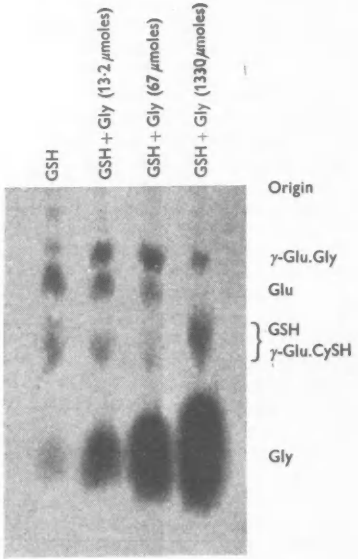

Fig. 6. Chromatogram showing effect of glycine concentration on the enzymic reaction with GSH. System: 0·2 ml. enzymic preparation (dialysed), 2 μmoles GSH and 0, 13·2, 67 and 1330 μmoles glycine, respectively. Solvent: propanol: water (80:20). Development: 60 hr. The highest concentration of glycine was obtained by weighing this amino acid into the incubation vessel.

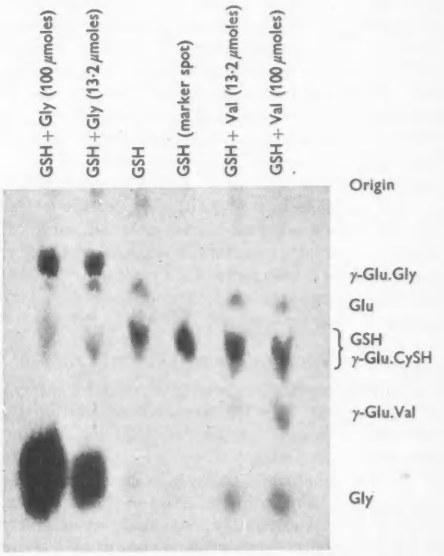

Fig. 7. Chromatogram showing effect of glycine and L-valine at two concentrations on the enzymic reaction with GSH. System: 0·2 ml. enzyme preparation (dialysed), 2 μmoles GSH and 0, 13·2 and 100 μmoles of glycine or L-valine, respectively. Incubation: 30 min. at 30° under N_2. Solvent: propanol: water (80:20). Development: 60 hr.

(3) *γ-Glutamyl peptide formation.* Some of the liberated cysteine reacted with GSH to form γ-glutamylcysteine and as such was not estimated. This might be the reason for the curve flattening off at about 80 % GSH breakdown (Fig. 5). The amount of cysteine liberated would therefore be greater than the amount estimated, and the latter cannot be a precise index of the reactivity of an amino acid. The error will be greatest with the most reactive amino acids, but would allow comparisons of reactivity to be made.

We have observed on chromatograms that an increase in the concentration of a reactive amino acid brought about the formation of additional γ-glutamyl peptide of the amino acid concerned and, by cysteine estimation, the release of extra cysteine. However, this is not true of L-valine and L-isoleucine. An increase in concentration of these amino acids led to a decrease in cysteine liberation (Fig. 4) but, as measured from chromatograms, corresponding γ-glutamyl peptide formation increased (Fig. 7). At a low concentration (132 μmoles/5 ml.), L-valine was less active than DL-norvaline, as measured by the formation of the corresponding γ-glutamyl peptide (Fig. 8). Similarly, L-isoleucine was less reactive than L-leucine and DL-norleucine in γ-glutamyl peptide formation. Qualitatively, the colour reactions of different γ-glutamyl peptides

Table 3. *The influence of* L-*valine on the liberation of cysteine from GSH by* L-*glutamine*

(System: as described in Experimental with 2 ml. enzymic preparation, 1 ml. containing 132 μmoles L-glutamine, 1 ml. water and 1 ml. solution containing 20·6 μmoles GSH. The valine was weighed directly into the vessel. Control: 2 ml. water in place of amino acids. Incubation: 30 min. under N_2 at 30°. Figures represent additional cysteine liberated, in excess of control, and are expressed in terms of % GSH breakdown.)

L-Valine (μmoles/5 ml.)	GSH breakdown in excess of control (%)
0	31
132	30
500	17
1000	3

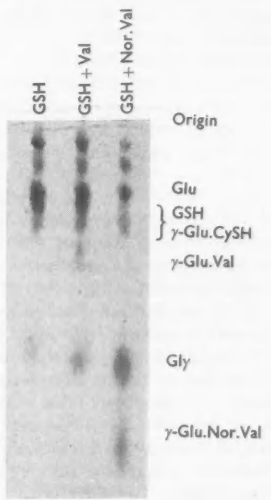

Fig. 8. Chromatogram showing effect of L-valine and DL-norvaline on the enzymic reaction with GSH. System: 0·2 ml. enzyme preparation (dialysed), 2 μmoles GSH and 13·2 and 26·4 μmoles L-valine and DL-norvaline, respectively. Incubation: 30 min. at 30° under N_2. Solvent: propanol: water (80:20) + 0·5 ml. ammonia (sp.gr. 0·880) in the chromatography jar. Development: 60 hr.

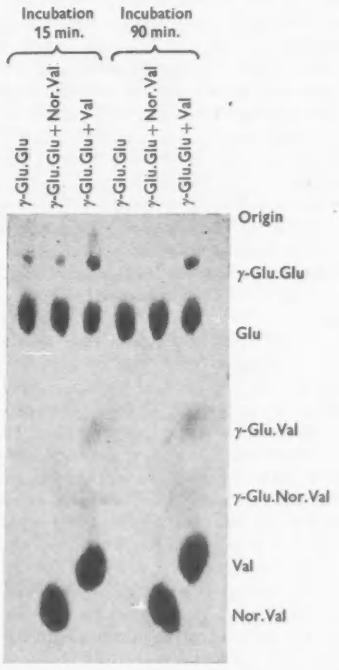

Fig. 9. Chromatogram showing effect of L-valine and DL-norvaline on the enzymic reaction with γ-L-glutamyl-L-glutamic acid. System: 0·2 ml. enzyme preparation (dialysed), 0·1 ml. solution containing 2 μmoles neutralized γ-L-glutamyl-L-glutamic acid, 0·2 ml. solution containing 13·2 and 26·4 μmoles L-valine and DL-norvaline, respectively. Incubation: 15 and 90 min. at 30° under N_2. Solvent: phenol saturated with water. Development: 20 hr.

with ninhydrin resembled the colour given by glutamic acid and not by the amino acid linked to it. Further, it would be expected that the reaction of γ-glutamyl peptides with ninhydrin would involve the free α-amino group of glutamic acid. It is, therefore, likely that differences in colour intensity of γ-glutamyl peptides on chromatograms indicate differences in amounts of these peptides. It seems justifiable then to conclude that at low substrate concentration L-isoleucine and L-valine are less reactive than their structural isomers, and that the figures given for the liberation of cysteine by the amino acids tested indicate their comparative reactivities.

The effect of increasing the concentration of L-valine which was added to a digest containing GSH and glutamine is shown in Table 3. At low substrate concentration, L-valine brought about very little reduction in cysteine liberated by L-glutamine, and this indicates that the apparent inertness of L-valine in the system GSH + valine was not due to inhibition of cysteinylglycinase by this amino acid. At high concentrations of L-valine, the liberation of cysteine by glutamine was reduced considerably. This reduction is consistent with: (i) L-valine inhibiting the attachment of the donor peptide, GSH, to the enzyme, (ii) L-valine, as a weak reactor, occupying the position of the acceptor amino acid on the enzyme and so reducing the number of transpeptidation reactions, (iii) L-valine, at high concentration, inhibiting cysteinylglycinase.

In a further test of the reactivity of valine, γ-L-glutamyl-L-glutamic acid was used instead of GSH, as it gave a simpler chromatographic pattern. The chromatogram (Fig. 9) showed that residual γ-L-glutamyl-L-glutamic acid was present in greater amounts in the presence of L-valine than in the presence of DL-norvaline. This result suggests that L-valine at low substrate concentration inhibited cysteine liberation for one or both of the reasons outlined in (i) and (ii).

(4) *Effect of concentration of acceptor amino acids.* Above a certain concentration characteristic of each added amino acid, cysteine liberation decreased. For the amino acids tested, but with the exceptions of L-aspartic acid, L-isoleucine and L-valine, the concentration of 132 μmoles/5 ml. represented a point on the concentration curves (Figs. 3, 4) on or below the point at which reactivity began to fall off. Such a concentration was therefore suitable to compare reactivities.

DISCUSSION

The γ-glutamyl transfer reaction has been shown to be catalysed by extracts of kidney and pancreas but not by extracts of liver tissue (Hanes *et al.* 1952). The distribution of γ-glutamyl transferase coincides with that of conjugase (Laskowski, 1950). Both enzymes are known to act on the γ-glutamyl linkage. Dakin & Dudley (1913) reported the presence of an antiglyoxalase in extracts of pancreas, and Woodward, Munro & Schroeder (1935) found that this enzyme was also present in extracts of kidney. The action of antiglyoxalase was found to be due to the destruction of GSH, the coenzyme in the glyoxalase system (Woodward *et al.* 1935; Salem & Crook, 1950). It is possible that these three enzymes are identical. Although there has been some tentative speculation about a possible role in protein synthesis (Fruton, 1950; Hanes *et al.* 1950, 1952) no function has been ascribed to the enzyme.

Unpublished observations by Hird, Neville & Springell have shown by use of differential centrifugation that γ-glutamyl transferase in sheep kidney and ox pancreas is particle bound and that by treatment with butanol (Morton, 1950) it can be liberated from the particles. Such preparations would still transfer the γ-glutamyl group to both water and amino acids.

Precise interpretation of the results obtained in the reaction of amino acids with GSH is difficult. However, using the indices established in the present investigation, broad comparisons can be made, especially within the groups of amino acids. Within the aliphatic series the most reactive amino acids were those with long unbranched side chains, e.g. methionine sulphoxide, glutamine and citrulline had the greatest reactivity; norleucine and norvaline had greater activity than leucine, isoleucine and valine. Amidation of the distal carboxyl groups of L-aspartic acid and L-glutamic acid, respectively, markedly enhanced reactivity. This has also been noted by Binkley (1952) and Fodor, Miller & Waelsch (1952).

The inhibitory effect of the β-methyl group in L-valine and L-isoleucine has previously been shown by other workers. Fox & Winitz (1952) found that these amino acids were also unreactive in the enzymic synthesis of anilides. Fox, Pettinga, Halverson & Wax (1950) have shown that benzoyl-L-valine amide is resistant to enzymic hydrolysis, and Smith, Spackman & Polglase (1952) have shown this for the amides of isoleucine and valine. These results are in contrast to those given by the corresponding derivatives of leucine. Valyl peptides are also resistant to hydrolysis by acid (cf. Sanger, 1952).

The preliminary reports by Binkley (1952) and Fodor *et al.* (1952), working with γ-glutamyl transferase from pig kidney, gave figures for several amino acids and their results are in agreement with those reported in the present paper.

In a previous publication (Hanes *et al.* 1952), it was found that L-arginine did not react with γ-L-glutamyl-L-glutamic acid to form the corresponding γ-glutamyl peptide. In the present work, however,

it has been shown that L-arginine is quite reactive with GSH. The reaction of L-arginine with GSH has also been reported by Kinoshita & Ball (1953).

Binkley (1951) and Binkley & Olson (1951) have given a special role to L-glutamine in GSH hydrolysis and suggest the following reaction mechanism:

Enzyme COONa + glutamine → Enzyme $CONH_2$ + Na glutamate,

Enzyme $CONH_2$ + GSH → Enzyme COOH + glutamine + cysteinylglycine.

The evidence for this hypothesis is that L-glutamine increases the 'hydrolysis' of GSH without itself being hydrolysed in the process. The results of these workers can be explained by the formation of γ-glutamylglutamine with the liberation of cysteinylglycine followed by hydrolysis of the latter. The production of a new peptide (almost certainly γ-glutamylglutamine) when L-glutamine is incubated with GSH has been reported (Hanes *et al.* 1952). In the present work L-glutamine has also been shown to be very reactive. The range of amino acids tested by Binkley (1949) and Binkley & Olson (1951) was not extensive and the smaller effects of other amino acids was thought to be due to the removal of inhibitory ions. As can be seen from Tables 1 and 2 there are many amino acids which are as active as L-glutamine and some even more so. These do not have the amide configuration. This means that the hypothesis of L-glutamine as a coenzyme in the hydrolysis of GSH lacks unequivocal supporting evidence.

SUMMARY

1. A modification of the method of Nakamura & Binkley (1948) for the estimation of cysteine in the presence of glutathione (GSH) is described.

3. Sheep-kidney γ-glutamyl transferase, which catalyses peptide-bond transfers between GSH and amino acids, has been investigated. With certain qualifications, cysteine liberated by these transfer reactions can be taken as an index of the comparative reactivity of amino acids in the system.

3. The relative reactivities of L-α-amino acids has been determined. The most reactive amino acids in the aliphatic series were those with long, unbranched side chains. The presence of a β-methyl group, as in L-valine and L-isoleucine, interfered with the reactivity of the amino groups of these amino acids. The ability of the amino groups of L-aspartic and L-glutamic acids to react with GSH was enhanced by amide formation as in L-asparagine and L-glutamine, respectively.

4. With the exceptions of L-aspartic acid, L-isoleucine and L-valine, an increase in amino acid concentration brought about an increase in the cysteine liberated from GSH. At higher concentrations reactivity fell off. Increases in the concentration of L-aspartic acid, L-isoleucine and L-valine caused a steady decrease in cysteine liberation.

5. Chromatographic evidence has now been obtained for transpeptidation with most of the common, naturally occurring amino acids, with the exceptions of L-aspartic acid, L-proline and L-hydroxyproline.

We wish to thank Mr A. M. Gallacher for preparing the glutathione.

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Some Errors in the Colorimetric Estimation of Oestriol, Oestrone and Oestradiol by the Kober Reaction

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In the course of an investigation of methods suitable for the accurate determination of oestriol, oestrone and oestradiol-17β in the urine of human subjects, it was found that the colour produced in the Kober reaction by oestrogens was depressed by 15–40% in the presence of solvent residues. The particular modification of the Kober (1931) reaction which was principally used in this investigation was one using quinol and aqueous sulphuric acid (Brown, 1952), but the same effect was found with the original phenol reagent of Kober under the conditions described by Venning, Evelyn, Harkness & Browne (1937). The inhibition of colour production may readily be demonstrated by the addition to a pure oestrogen of the residue from distillation of ether which has been used to extract dilute mineral acid and washed with bicarbonate and water (as is required in the initial extraction of oestrogens from hydrolysed urine). The effect persisted even when the ether was purified by a variety of methods. The magnitude of error involved was sufficient to necessitate a thorough re-investigation of the colour reaction described by Brown (1952).

This reaction, like all modifications of the Kober method, is a two-stage process, in which the initial formation of a yellow compound with a green fluorescence is followed by its conversion into a pink, non-fluorescent complex on reheating in more dilute sulphuric acid. This pink colour fades if heated in still more dilute solutions. There are thus three possible forms of interference with the colour produced, namely: (1) failure to form the initial yellow complex (inhibition type I); (2) failure to convert the yellow complex fully into the pink (inhibition type II); (3) instability of the pink complex (fading).

METHODS

Purification of materials. Ethanol (absolute) was purified by refluxing for 12 hr. with 5% (w/v) Zn dust and 5% (w/v) NaOH pellets, distilling, standing over *m*-phenylenediamine for at least 7 days and then distilling twice. The ether (A.R. grade) was purified as follows: 2 l. were shaken for 6 min. with 16 g. of $AgNO_3$ in 120 ml. of water and 200 ml. of N-NaOH (Werner, 1933), then washed with water and distilled.

The $NaHCO_3$ used was A.R. Two types of A.R. H_2SO_4 were used: type *A* was manufactured by British Drug Houses Ltd., type *B* by Hopkin & Williams Ltd.

Apparatus. Pyrex test tubes (18 × 150 mm.) were used initially for the colour reaction. The later experiments were done in Quickfit & Quartz test tubes (23 × 150 mm.) with *B*. 19 ground-glass sockets fitted with cones to which was attached glass tubing (5 mm. diam.) bent at an acute angle. These 'Kober tubes' were introduced to minimize changes in the volume of solution from condensation or evaporation of water during the heating stage.

Glassware was cleaned with chromic acid, rinsed with water and ethanol, washed with a vigorous stream of water for 2–3 min., and finally rinsed with distilled water.

In the preliminary experiments, optical densities were measured by a Spekker absorptiometer in 1 cm. cuvettes with Ilford Spectrum Green, Violet and Yellow (604, 601, 606) filters (transmission maxima at 520, 430 and 580 mμ., respectively).

Absorption spectra and the colours of solutions obtained by the method finally chosen were measured in a Unicam diffraction-grating spectrophotometer (SP 600).

Preparation of oestrogen solutions. Standard solutions of pure crystalline oestrogens were prepared in ethanol (5 mg./100 ml.), the appropriate volumes were added to the reaction tubes and the solvent was removed by heating at 90–95° in a current of air. With aldehyde-free ethanol, no loss could be demonstrated by this procedure. The solutions were stored at 4°.

In order to demonstrate the effect of solvent residues, pure oestrogens were extracted with ether (1 × 150 ml., 3 × 125 ml.) from a mixture of 500 ml. of distilled water and 75 ml. of 30% (v/v) type *B* H_2SO_4. The combined ether extracts were washed with 8·5% (w/v) $NaHCO_3$ (3 × 35 ml.) and water (2 × 25 ml.). This corresponds to the initial stage of the method for the estimation of oestrogens in urine. The residues obtained after distillation of the ether were transferred with aldehyde-free ethanol to the tubes for the colour reaction. The solvent was removed either by heating in a stream of air or under reduced pressure in a stream of N_2, no significant difference between the two methods being demonstrable.

Oestrogen residues prepared by these methods are referred to as 'standard' and 'extract', respectively, throughout this paper.

Colour reagents and reaction. These were based on the conditions described by Brown (1952). The dilutions of H_2SO_4 employed for preparation of Kober reagents were 76, 66 and 60% (v/v) for oestriol, oestrone and oestradiol-17β, respectively. The term 'heating at 100°' means heating in a boiling-water bath.

Colour-correction formulae. Since solvent residues contribute a non-specific background colour in the reactions, it was necessary to correct for this enhancing effect. For measurements in the absorptiometer, an equation, devised by Dr J. B. Brown (to be published), was used.

$$D_{corr.} = (4D_{604} - 3D_{606} - D_{601})/3{\cdot}8,$$

where $D_{corr.}$ is the optical density corrected for the non-pink components, and D_{604}, D_{606}, D_{601} are the observed optical densities with the Ilford filters indicated by the subscripts. For corrections of measurements in the spectrophotometer, as used in the final method, the equation of Allen (1950) was applied.

RESULTS

Type I inhibition. Failure to form initial yellow complex

Typical examples of the incomplete recoveries obtained by extracting oestriol (25 μg.) from dilute mineral acid with ether are shown in Table 1*A*. The oestrogen content was determined by heating the extraction residue at 100° for 20 min. with 4 ml. of 2% quinol in 76% (v/v) sulphuric acid, diluting with 1 ml. of water, reheating for 5 min., and diluting to 15 ml. with a solution comprising 70 ml. water and 30 ml. H_2SO_4 (Brown, 1952). The increase in non-specific background colour and the depression of pink are evident. Addition of certain reducing agents to the reagent immediately before use improved the recovery values. The results obtained with *m*-cresol are shown in Table 1*B*.

Sulphonation of the reagent as cause of type I inhibition. These findings suggested that the reagent had insufficient reducing power in the presence of oxidants in the solvent residues. Pinnow's (1915, 1917) work suggests that quinol would be almost quantitatively sulphonated, and thus its reducing power diminished, during the preparation and ageing of the reagent. Pinnow's experiments were repeated in part, and it was found that a reagent prepared by dissolving 2 g. of quinol in 100 ml. of 76% (v/v) sulphuric acid at room temperature contained more than 90% of the phenol unchanged at 24 hr. but less than 2% after 3 weeks. Moreover, solution of the quinol by heating to 35–40° and 70–80° caused sulphonation to the extent of 50 and 97%, respectively. It seemed that the yields of oestriol (determined colorimetrically) obtained by extraction from aqueous mineral acid with ether might vary with the method of preparation and age of the reagent. The results of experiments demonstrating this are shown in Table 2. No *m*-cresol was added, and the reaction was carried out as described by Brown (1952).

An attempt was made by substitution of duroquinol (2:3:5:6-tetramethylquinol) for quinol to obtain a reagent which would be stable on ageing, but the duroquinol was found to be too strong a reducing agent since oxidation to intensely coloured products occurred merely on heating in 76% (v/v) sulphuric acid in absence of oestrogen.

In the absence of solvent residues, the reducing power of the sulphonated quinol appears to be adequate for satisfactory colour development. However, for the estimation of oestriol in the presence of oxidizing contaminants such as may arise from urine or solvent residues, the reagent must contain free quinol. This requirement is not met in reagents prepared and used as suggested by Brown (1952).

Comment. Chamot & Pratt (1909, 1910) found irregular results in the colorimetric determination of nitrates owing to changes in the composition of their phenol:sulphuric acid reagent. Clayton (1949) noted that the best results in the determination of oestriol in the presence of solvent residues were obtained with a reagent prepared as suggested by Venning *et al.* (1937) if used within 48 hr. Marlow (1950) also used a freshly prepared reagent.

Table 1. *Effect of ether residues on the development of colour by oestriol* (25 μg.) *in the quinol:sulphuric-acid reaction*

(In this and subsequent tables recovery values are 100 $D_{corr.}$ for extract/$D_{corr.}$ for standard.)

Expt.	Oestriol used	D_{604}	D_{601}	D_{606}	Recovery (%)
		A. No *m*-cresol added			
1	Standard	0·160	0·047	0·002	—
	Extract	0·118	0·058	0·018	61
2	Standard	0·168	0·028	0·004	—
	Extract	0·125	0·059	0·025	57
3	Standard	0·177	0·032	0·004	—
	Extract	0·121	0·055	0·018	57
	B. *m*-Cresol (0·1 ml.) added to each sample at start of colour reaction				
1	Standard	0·150	0·033	0·0	—
	Extract	0·168	0·060	0·025	94
2	Standard	0·165	0·022	0·0	—
	Extract	0·192	0·081	0·038	90
3	Standard	0·165	0·028	0·003	—
	Extract	0·176	0·081	0·030	87

Table 2. *Effect of age and method of preparation of reagent on colour production in the presence of ether residues by oestriol (25 μg.)*

(Reagent 1: quinol (2 g.) dissolved at room temperature in 100 ml. of 76 % (v/v) H_2SO_4 and used within 12 hr. Reagent 2: as for reagent 1; but heated to 40–50° during solution of quinol. Reagent 3: as for reagent 1, but 3 weeks old.)

Type of reagent	Oestriol used	D_{604}	D_{601}	D_{606}	Recovery (%)
1	Standard	0·180	0·032	0·004	—
	Standard	0·186	0·033	0·005	—
	Extract	0·179	0·053	0·018	89
	Extract	0·173	0·061	0·019	84
1	Standard	0·179	0·031	0·004	—
	Standard	0·175	0·025	0·0	—
	Extract	0·165	0·052	0·013	85
	Extract	0·174	0·068	0·019	85
2	Standard	0·179	0·035	0·0	—
	Extract	0·128	0·040	0·009	65
	Extract	0·143	0·047	0·006	74
3	Standard	0·192	0·048	0·0	—
	Standard	0·190	0·048	0·0	—
	Extract	0·140	0·078	0·029	55
	Extract	0·150	0·089	0·031	58
	Extract	0·157	0·088	0·032	60
	Extract	0·151	0·088	0·032	59

Ageing of the reagent did not interfere with colour production by oestrone or oestradiol-17β in the presence of solvent residues. This was to be expected for oestrone, since Brown (1952) showed that this oestrogen does not require a reducing agent in the first stage of the Kober reaction. Consideration of the findings of previous workers suggests a possible explanation for the anomalous behaviour of oestriol.

It appears that oestriol when heated in sulphuric acid can undergo at least two different reactions. The first is apparently an oxidation and is not undergone by oestrone (Marrian, 1938). The second, the Kober reaction, requires a reducing agent and is undergone much less readily than by oestrone (Cartland, Meyer, Miller & Rutz, 1935; Bachman, 1939; Brown, 1952). The fact that oestriol requires longer heating and higher acid concentration led Szego & Samuels (1940) to suggest that in the first stage of the Kober reaction, oestriol is dehydrated to oestrone, a possibility which is supported by the similarity of the absorption spectra of the two coloured products (cf. Fig. 5). The fact that 16-epioestriol (oestra-1:3:5(10)-triene-3:16β:17β-triol) gives a more intense colour under these conditions than does oestriol (Marlow, 1950) is also suggestive.

It is possible that in the Kober reaction for oestriol, a reducing agent is required to prevent the alternative oxidative reaction, thus permitting quantitative dehydration to oestrone. Under these circumstances it would be expected that oxidants arising from solvent residues might tend to prevent full formation of the yellow precursor of the Kober pink (type I inhibition), and that the addition of an effective reducing agent would exert a stabilizing action.

Type II inhibition (failure to convert yellow into pink colour) and fading

In agreement with Brown's (1952) results, it was found that concentrations of 76, 66 and 60 % (v/v) of sulphuric acid were the most satisfactory ones for the first stage of the reaction with oestriol, oestrone and oestradiol-17β, respectively. That a concentration of 60 % was the best for the second stage could not, however, be demonstrated (see Fig. 1). Samples of oestriol (25 μg.) were heated for 20 min. at 100° with 4 ml. of 2 % quinol in 76 % (v/v) sulphuric acid. The cooled solutions were diluted with either 1 ml. or 2 ml. of water to give second-stage concentrations of approximately 60 % or 50 %, respectively, mixed, reheated for 0–10 min., and diluted to a final concentration of 40 % (15 ml.). Optical densities were measured with the Ilford 604 and 601 filters after 30 min. and again after 90 min.

Fig. 1 shows that dilution with 1 ml. of water and 5 min. reheating were insufficient to cause full conversion of yellow into pink (low D_{604} value), prolonged standing at room temperature being required. Dilution with 2 ml. of water to give a second-stage concentration of approximately 50 % permitted complete development of colour after reheating for 5 min., but the pink tended to fade on dilution of the solution.

It was observed that the development of colour was aided by sunlight and it was considered that

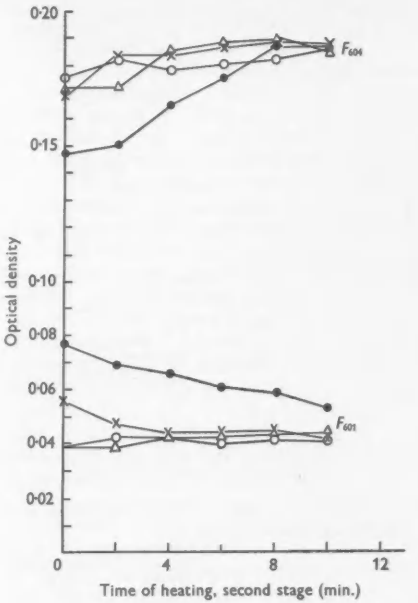

Fig. 1. Effect of variation of H_2SO_4 concentration of the second stage, and of standing at room temperature after final dilution to 40%, on intensity of colour produced by oestriol (25 μg.). Second-stage concentration of 60% (diluent, 1 ml. of water): ●—●, after 30 min. standing; △—△, after 90 min. standing. Second-stage concentration of 50% (diluent, 2 ml. of water): ×—×, after 30 min. standing; ○—○, after 90 min. standing.

In this and subsequent figures, the symbols F_{604} and F_{601} denote the Ilford filters used in the measurement of optical density.

Table 3. *Effect of variation of* H_2SO_4 *concentration of second stage and of illumination on stability of final colour intensity obtained with oestriol* (25 μg.)

Time of reading (min.)	2nd stage concn. 60%		2nd stage concn. 50%	
	D_{604}	D_{601}	D_{604}	D_{601}
A. Solutions exposed to sunlight, in a brightly lit laboratory during the development of the colour, at all stages except during heating				
5	0·170	0·044	0·151	0·048
15	0·160	0·042	0·148	0·036
90	0·153	0·043	0·140	0·025
120	0·153	0·041	0·132	0·030
B. Reactions carried out at night in dimly lit laboratory				
5	0·178	0·079	0·198	0·059
15	0·189	0·072	0·200	0·053
90	0·194	0·065	0·199	0·049
120	0·198	0·057	0·199	0·048

differences in illumination in the two laboratories might account for the discrepancy between the results reported here and those of Brown (1952). The results of an experiment demonstrating this effect are shown in Table 3. The colour reaction was carried out as in the experiment shown in Fig. 1, except that the period of reheating in all cases was 5 min. The data are typical of a large series of experiments conducted with oestriol, oestrone and oestradiol-17β with reagents both freshly prepared and aged. It will be seen that either a decrease in the second-stage concentration or an increase in illumination was required for full conversion of yellow into pink, as shown by low D_{601} values. When both these conditions were present, there was a marked tendency to a fading of the pink.

Comment. The conversion of yellow into pink in the second stage of the Kober reaction is apparently an oxidation, since it is aided by ferric ions (Haenni, 1950), hydrogen peroxide (Brown, 1952) and cupric ions (present investigation). If the oxidizing power of the system is too small, there will be a tendency for incomplete conversion of yellow into pink in the second stage, rendering the reaction susceptible to interference by contaminants arising from the extraction procedure. Reactions such as those described by Bachman (1939) and Brown (1952), in which the completion of the colour reaction occurs at room temperature after final dilution, may be unstable because of this effect. Brown (1953) found that the time required for complete formation of the pink compound was altered by urinary residues.

On the other hand, it has long been known that the pink may be converted into a colourless compound by hydrogen peroxide (Kober, 1931). Thus, when conditions are such as to facilitate the conversion of yellow into pink, the reaction will be susceptible to fading. Cohen & Marrian (1934) found this to occur in the presence of urinary residues and corrected it by increasing the sulphuric-acid concentration of the second stage. The effect of sunlight in modifying the conditions of the reaction might have been expected from the known photosensitivity of the sulphuric-acid colour reaction for cholesterol (cf. Kenny, 1952) and in this connexion it is perhaps significant that Szego & Samuels (1943) kept the solutions in the dark at one stage of their modified Kober reaction.

Elimination of interference with colour production in the Kober reaction

Elimination of type I inhibition. In order to avoid the inconvenience of using freshly prepared reagent, quinol was added at the start of the colour reaction.

Elimination of type II inhibition. Brown (1952) showed that the pink colour obtained with pure oestrogen was stable at 100° for 2 hr. at a concentration of 60% (v/v) sulphuric acid. Accordingly, it

was decided to eliminate the final dilution, thereby minimizing the possibility of fading and doubling the sensitivity of the reaction. The solutions were protected from bright light at all stages. The optimum sulphuric acid concentration for the second stage was reinvestigated.

The appropriate quinol:sulphuric acid reagent (4 ml.) was heated at 100° with eleven samples of 25 μg. of oestrogen in Kober tubes for 20 min. The solutions were cooled, diluted with 3·5 ml. of sulphuric acid of various concentrations, and reheated, samples being withdrawn at 2 min. intervals from 0 to 20 min. In all cases the final colour was stable at room temperature for at least 12 hr. The results obtained with oestrone and its reagent (2 % quinol in 66 % (v/v) sulphuric acid) are shown in Fig. 2.

With a second-stage concentration of 60 %, prolonged heating was required to effect full conversion of yellow into pink, whereas with 40 %, prolonged heating caused fading. Similar results were obtained with oestriol and oestradiol-17β. In all cases the best compromise between the two opposing tendencies to type II inhibition and fading was obtained with second-stage concentrations of 50–55 %. When the colour reaction was carried out in test tubes (18 × 150 mm.), it was found that continual stirring during the reheating increased the pink, but did not alter the relationship between time of heating and sulphuric acid concentration. The use of the larger bore Kober tubes gave optimum D_{604} values when the usual procedure of shaking the tube 5–10 times before reheating was adhered to.

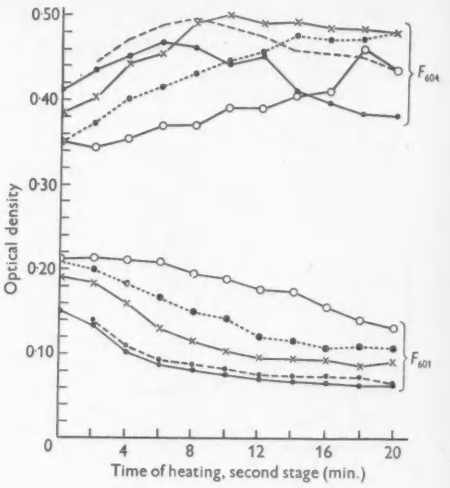

Fig. 2. Effect of variation of H_2SO_4 concentration of the second stage (oestrone 25 μg.).

	Diluent, H_2SO_4 (%, v/v)	Second-stage concn. (%)
●—●	10	40
●– – –●	20	45
×—×	30	49
●. . .●	40	54
○—○	53	60

Variation of colour reaction with type of sulphuric acid

The experiments described above were conducted with reagents made from type *B* sulphuric acid. There are some indications in the literature that the make of sulphuric acid used profoundly affects the course of the Kober reaction (cf. Engel, Slaunwhite, Carter & Nathanson, 1950; Venning, 1952). Before the conditions of the present colorimetric method were standardized, two types of sulphuric acid were compared. The results of oestradiol-17β with a second-stage concentration of 60 % are shown in Fig. 3. The marked difference in optical densities obtained with reagents made from types *A* and *B*, as well as the strikingly different rates of colour formation, is apparent. It was clear that these findings rendered the colour method unsatisfactory.

The effect of the addition of trace amounts of oxidizing agents to the diluting acid is also shown. The indication is that in neither reagent were conditions optimum; trace contaminants could cause falsely high results.

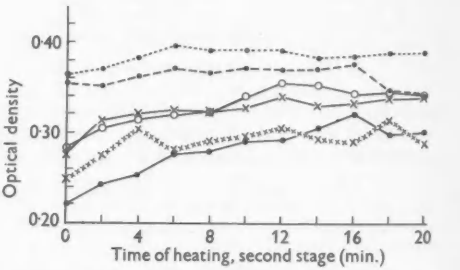

Fig. 3. Effect of type of H_2SO_4 used in preparation of reagent, and of oxidants added in the second stage, on the colour intensity produced by oestradiol-17β (25 μg.).

	H_2SO_4 type	Additions in diluent (mg./100 ml.)
●—●	*B*	Nil
×—×	*B*	Cu^{2+} (8·0)
○—○	*B*	NO_3^- (0·1)
× × ×	*B*	Fe^{3+} (3·5)
●– –●	*A*	Nil
●. . .●	*A*	Cu^{2+} (8·0)

Variation of reagent with type of sulphuric acid

The effects of the addition of trace amounts of an oxidant (hydrogen peroxide) are shown in Table 4. The reaction was carried out by heating 4 ml. of 2% (w/v) quinol in 60% (v/v) sulphuric acid (type *B*) with 50 μg. of oestradiol-17β at 100° for 20 min., diluting with 3·5 ml. of 60% (v/v) sulphuric acid (type *B*) and reheating for 15 min. Greater intensities were produced when the oxidant was added in the first stage of the reaction and when the reagent was freshly prepared, suggesting that the oxidizing agents were acting indirectly by an oxidation of the quinol. With ageing of the reagent, the quinol was mostly sulphonated, more resistant to oxidation (Pinnow, 1917) and less effective in development of colour. Moreover, it was preferable to have the oxidized form present in the first stage of the reaction.

Table 5 shows typical results obtained with reagents containing varying amounts of quinol and its oxidation products. The solvent in all cases was 60% (v/v) sulphuric acid (type *B*) and the reaction was as described for the experiment shown in Table 4. It is apparent from reagents 1–3 that a poised system of quinol:quinone is not desirable, a large excess of the former being required for maximum colour production. The difference in behaviour of reagents 4 and 5 is not readily explained. The increased colour found with reagent 5 has been repeatedly confirmed during the past year, not only for the oestradiol-17β reagent but also for the reagents for oestrone and oestriol. There is a marked difference in appearance: reagent 4 is colourless, reagent 5 pink. It is possible that the effective agent is an oxidation product intermediate between quinol and quinone. Solutions prepared as for reagent 5 are subsequently referred to as 'modified reagents', while those prepared by dissolving 2 g. quinol in 100 ml. aqueous sulphuric acid are referred to as 'pure reagents'.

Comparison of pure and modified reagents. Fig. 4 compares the effectiveness of pure and modified reagents, prepared from different types of sulphuric acid, in the formation of colour with oestriol. Water was used as the diluent. Preliminary experiments established that the concentration of approximately 55%, which has been used in the second stage for both the pure reagents, was required in order to permit full conversion into pink in 15–20 min. heating with type *B* sulphuric acid. These conditions were imperfect, and the colour was liable to be enhanced by trace contamination. No significant change occurred on ageing. Application of the same conditions to a pure reagent prepared from type *A* sulphuric acid gave maximum optical densities in 5 min. heating and a tendency to fading. It is clear then that 'pure' (unmodified) reagents can be used only when the conditions for the second stage of the reaction are developed for each type (and possibly each batch) of sulphuric acid.

Table 4. *Quinol:H_2SO_4 reaction with oestradiol-17β (50 μg.) in the presence of H_2O_2 (50 μg.): effect of time of addition of H_2O_2 and age of reagent*

	D_{604}	D_{601}
H_2O_2 (50 μg.) added in first stage		
5-week-old reagent	0·682	0·161
	0·697	0·192
Fresh reagent	0·770	0·165
	0·762	0·166
H_2O_2 (50 μg.) added in second stage		
5-week-old reagent	0·613	0·190
	0·698	0·176
Fresh reagent	0·666	0·169
	0·687	0·170
No H_2O_2 added		
5-week-old reagent	0·558	0·208
	0·551	0·208
Fresh reagent	0·611	0·201
	0·620	0·206

Table 5. *Effect of quinol and its oxidation products on the colour produced by oestradiol-17β (50 μg.) in 60% (v/v) H_2SO_4*

(Reagent 4 was prepared by mixing solutions of quinol and quinone; reagent 5 was prepared by adding quinol to a solution of quinone.)

Reagent no.	Quinhydrone (mg./100 ml.)	Quinol (mg./100 ml.)	Quinone (mg./100 ml.)	D_{604}	D_{601}
1	2	—	—	0·553	0·243
				0·553	0·243
2	2	500	—	0·638	0·174
				0·658	0·179
3	2	2000	—	0·694	0·170
				0·684	0·170
4	—	2000	2	0·685	0·183
				0·674	0·192
5	—	2000	2	0·775	0·159
				0·761	0·162

The 'modified' reagent prepared from type *A* or type *B* sulphuric acid gave maximum optical densities and no tendency to fading or type II inhibition with a second-stage concentration of approximately 60%. (Virtually identical results were obtained with the two types of sulphuric acid.) As the reagent aged, there was slight diminution (3–5%) in the formation of pink and a corresponding increase in the residual yellow. This was eliminated, as shown, by the addition of 100 mg. of quinol in the second stage of the reaction.

Exactly comparable results were obtained with pure and modified reagents for oestrone and oestradiol-17β. The stability of the reagents in the presence of solvent residues is attested by the typical experiments in Table 6.

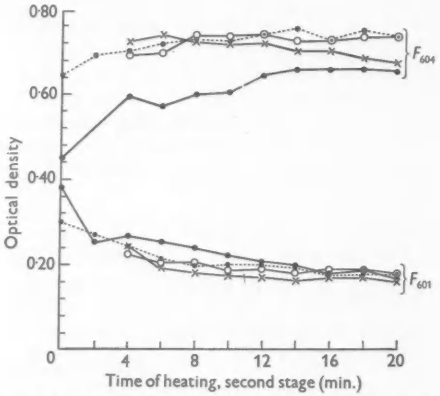

Fig. 4. Development of colour by oestriol (50 μg.) with pure and modified reagents. ●—● Pure reagent, type *B* H_2SO_4; ×—×, pure reagent, type *A* H_2SO_4; ●···●, modified reagent, 1-day old; ○—○, modified reagent, 10-days old, quinol (100 mg.) added at second stage.

Table 6. *Recovery of oestrogens* (25 μg.) *on extraction from aqueous* H_2SO_4 *with modified reagents and colour reaction*

Oestradiol-17β	D_{604}	D_{601}	D_{606}	Recovery (%)
Extract	0·362	0·087	0·010	100
	0·365	0·092	0·014	100
Standard	0·345	0·070	0·0	—
	0·349	0·071	0·0	—
Oestrone				
Extract	0·445	0·108	0·009	98
	0·452	0·110	0·013	99
Standard	0·444	0·087	0·002	—
	0·442	0·090	0·003	—
Oestriol				
Extract	0·393	0·170	0·042	92
	0·400	0·188	0·050	90
Standard	0·385	0·110	0·013	—
	0·390	0·112	0·016	—

An improved method for the colorimetric determination of oestriol, oestrone and oestradiol-17β

Since residues from the phenolic fraction of urine give a background colour which is not linear over the wavelength range covered by the 601 (violet) and 606 (yellow) filters, optical densities are now determined over a narrower range in the Unicam SP-600 instrument which permits measurement of 3·3 ml. of solution in a cell of 1 cm. optical thickness. The volumes of solution have been modified to suit these new conditions, the final reaction being described below.

Preparation of reagents

Reagent for oestradiol-17β. $NaNO_2$ (A.R. grade, 10 mg.) was added to 1 l. of H_2SO_4 (A.R., 60%, v/v) and 20 mg. quinone were dissolved with warming (types *A* and *B* acid give similar results). The solution turned yellow, changing to a light green with a definite opalescence. Quinol (20 g. of B.D.H. Laboratory Reagent) was added and dissolved by warming and shaking. The solution lightened in colour, becoming pale pink but still opalescent. After filtration through sintered glass (porosity no. 4, fine) the reagent was stored in an amber bottle. Occasionally opalescence reappeared with ageing and a second filtration was required. Certain batches of H_2SO_4 caused the appearance of brown oxides of nitrogen in small quantities during the dissolution of the quinol. These were removed during the filtration. At this low concentration of H_2SO_4, the quinol occasionally crystallized on cooling and had to be reheated.

Reagent for oestrone. This reagent was prepared as above but with 66% (v/v) sulphuric acid as the solvent. The final reagent was yellow with a trace of pink.

Reagent for oestriol. This was prepared similarly to the oestradiol-17β reagent but with 76% (v/v) sulphuric acid as the solvent. Less opalescence formed and the final solution was yellow with no trace of pink.

Satisfactory reagents can be prepared exactly as recommended by Brown (1952) with certain batches of type *A* H_2SO_4 provided quinol is added to the oestriol reagent before use and provided all reagents are allowed to stand at room temperature for at least 1 week before use. Such reagents are highly coloured, being pink, yellowish pink, and yellow, for oestradiol-17β, oestrone, and oestriol, respectively. Satisfactory reagents cannot be prepared in this manner from type *B* H_2SO_4.

Colour production. The appropriate reagent (2·6 ml. for oestradiol-17β and oestriol, 3·0 ml. for oestrone) was added to dry residues of oestrogens in Kober tubes. Quinol (50 ± 5 mg. from a calibrated measure) was added and the tubes, with tops in place, were heated for 20 min. in a bath of boiling water, with shaking after 2 min. and 5 min. to ensure adequate mixing and the dissolution of the quinol. The solutions were then cooled in a bath of cold water (approx. 15°), 50 mg. of quinol were added and diluted as follows: oestradiol-17β, with 0·7 ml. of reagent; oestriol, with 0·7 ml. of water; oestrone, with 0·3 ml. of water. The tubes were then shaken 5–10 times and reheated, with the tops in

place, for 15 min. with two shakings during this period to ensure dissolution of the quinol.

Measurement of optical densities. The cooled solutions (which are stable for several hours) were transferred to 1 cm. cuvettes and their optical densities measured at 480, 512·5 and 545 mμ. for oestrone and oestriol and at 480, 515 and 550 mμ.

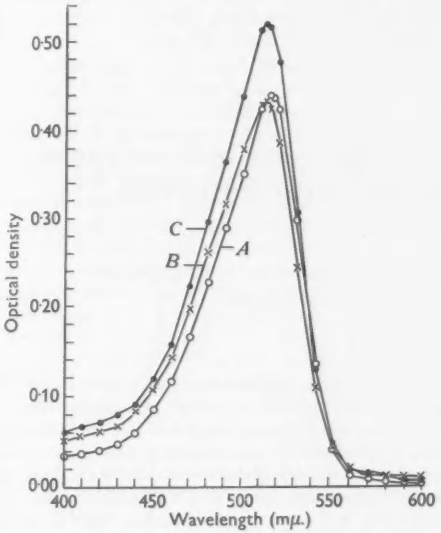

Fig. 5. Absorption spectra of the first coloured products obtained from pure oestrogens (10 μg.) with the modified reagents and reactions. *A*, oestradiol-17β; *B*, oestriol; *C*, oestrone.

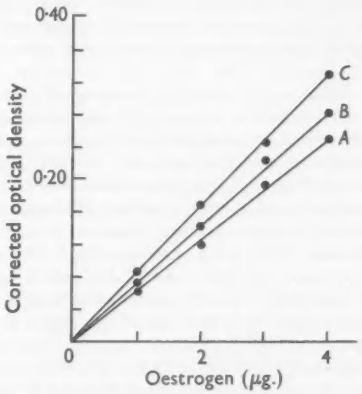

Fig. 6. Relationship between concentration of oestrogen and corrected optical density with the modified reagents and reactions. *A*, oestriol; *B*, oestradiol-17β; *C*, oestrone.

for oestradiol-17β. The colour correction formula of Allen (1950) was then applied.

Characteristics of the colour reaction. The absorption spectra of the final coloured products are shown in Fig. 5 ($\lambda_{max.}$, 512·5 mμ. for oestrone and oestriol, 515 mμ. for oestradiol-17β). The sensitivity, as shown by $E^{1\%}_{1\,cm.}$, is approximately 1700 for oestrone and 1400 for oestriol and oestradiol-17β. Proportionality between concentration of oestrogen and corrected optical density is shown in Fig. 6.

SUMMARY

1. Solvent residues interfere with the production of colour by oestriol, oestrone and oestradiol-17β in the reaction with the quinol:sulphuric acid reagents of Brown (1952). There are three different kinds of interference with the colour reaction.

2. The first of these (failure to form the initial yellow complex; type I inhibition) applies only to oestriol and is due to diminished reducing power of the reagent as a result of sulphonation of the quinol. This has been overcome by the addition of quinol immediately before colour development.

3. The other two types of interference depend upon the fact that the second stage of the Kober reaction, involving a conversion of the yellow complex into pink, appears to be an oxidation which, if excessive, causes fading. Under the conditions described by Brown (1952) the conversion of yellow into pink and the stability of the latter are influenced by the degree of illumination of the laboratory, size of the tube used and degree of stirring. Elimination of the final dilution stage, the use of larger-bore tubes and modification of the conditions for the second stage removed these variable factors.

4. The optimum reaction conditions depend upon the make of analytical reagent grade sulphuric acid used; this variable can be eliminated by inclusion of trace amounts of sodium nitrate and quinone in the reagent.

5. The modified reaction is unaffected by solvent residues and gives colour intensities which are reproducible and obey Beer's law over a suitable range of concentration.

The author wishes to thank Prof. G. F. Marrian, F.R.S., for his supervision of the investigation, and Mr James Gordon for skilled technical assistance. Part of the expenses of this work were defrayed from Medical Research Council funds.

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Continuous Direct Photometry of Dyed Materials in Filter Paper with Special Reference to the Estimation of Proteins Separated by Electrophoresis

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The estimation of protein fractions after their separation by electrophoresis in filter paper and subsequent staining may be carried out by excision, elution, and colorimetric estimation (Cremer & Tiselius, 1950) or by direct photometry, either intermittent (Grassmann, Hannig & Knedel, 1951) or continuous. Direct photometric methods have the advantage of being less laborious and also of requiring less material.

A brief account of an apparatus and technique for direct continuous photometry has already been given (Crook, Harris & Warren, 1952). In the present paper the underlying problems involved in such a technique are discussed, and a detailed account is given of an apparatus which has given satisfactory results in practice.

CONSIDERATIONS IN DESIGN

In designing an apparatus and a technique of measuring continuously the optical-density distribution in a strip of filter paper, many decisions have to be made between possible alternative solutions of the problems that arise. Some of these are straightforward, such as the choice of moving part, since it is obvious that movement of the paper past a fixed optical system and photocell is the simplest arrangement. Similarly, although a cylindrical paper carriage is more compact, a flat carriage is much simpler to construct and to retain the paper upon.

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At first sight, measurement of the amount of colour by reflexion from the dry paper might appear preferable to the measurement of transmission, since it avoids all the complications of the scattering of light by the fibres of the paper and the consequent necessity for an immersion fluid. However, although it is easy to perceive qualitative differences in reflexion by the unaided eye, preliminary experiments showed that there were quantitative irregularities in the amount of light reflected, due presumably to variations in the surface texture of the paper and to differences in the degree of penetration of protein and dye into the paper. Thus a transmission system which allows the light to pass through all the dyed protein is the only admissible type.

The paper must, however, be rendered as translucent as possible to reduce light scattering, i.e. it must be made to approach an optically homogeneous medium by the use of an immersion fluid. Theoretically, except for a small residual effect due to differences in the dispersion of immersion fluid and paper which would cause the combination to act as a Christiansen filter, an immersion fluid with a refractive index equal to that of the paper would reduce the scatter to zero provided the paper was homogeneous. However, since the paper is not homogeneous and scatter can only be reduced to a minimum, the refractive index of the immersion medium is not critical provided it is in the neighbourhood of 1·55, which is the approximate refractive index of cellulose. On trial, media with refractive indices in this region were found to be

sufficiently effective in minimizing scatter in filter paper (Whatman no. 1). It is, of course, important to remove all air trapped in the interstices of the paper.

The presence of residual scattering makes it necessary to consider carefully the layout of the optical system. The fact that the photocell must 'see' only a restricted length of paper requires the introduction of a slit parallel to the short axis of the paper strip. The best restriction is obtained by a narrow slit very close to the paper on the photocell side, since this prevents scattering of light into the photocell from adjacent areas of the paper. However, variations in scattering power of the paper then appear as variations in optical density and are recorded by the photocell as though they were variations in the amount of protein. Such variations may be sufficiently serious to make this positioning of the slit undesirable. It therefore appeared preferable to use the slit to restrict the illuminated area. By the use of a broad photocell placed close to the paper most of the scattered light is picked up. Variations of the scattering power of the paper become of minor importance and the system measures the light absorbed irrespective of the scattering. A small residual effect remains owing to differences in absorption within the paper of obliquely scattered light, according to whether scattering takes place through a clear or through a dyed region. This tends to depress the transmission of a clear region surrounded by heavily dyed areas and vice versa. This is a disadvantage of having the slit between the light source and the paper, and is obviated by having the slit immediately in front of the photocell. However, this effect was less important than variations in the scattering power of the paper.

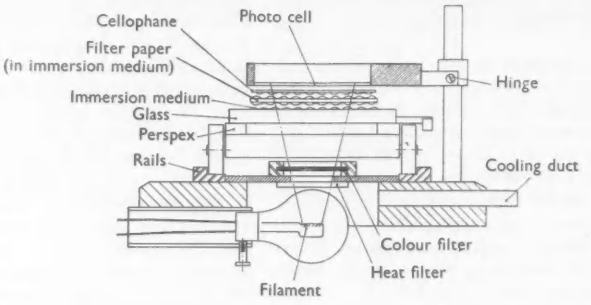
Correct restriction of the area of the paper 'seen' by the photocell demands a sharply outlined image of the slit. Such a requirement, combined with high light intensity, demands an elaborate optical system of condenser and focusing lenses for its complete realization. The next best solution is to remove lenses altogether from the system, relying on minimizing the distance between source and paper to obtain maximum intensity and placing the slit as close as possible to the paper to reduce the penumbra to a minimum. Such a system has the advantage of simplicity and compactness.

The restriction of the illumination by the slit makes inevitable the amplification of the photocell output before it is available to work a recording milliameter, even if a photomultiplier is used. The choice of photocell type therefore depends upon the available amplifiers, the necessary sensitivity, and the geometry of the optical system. The last two requirements are best satisfied by a selenium barrier layer cell, because of its high sensitivity compared with the simple vacuum and gas-filled emission cells and because its large sensitive area and absence of glass envelope make it possible to bring the sensitive surface close to the paper. It is generally regarded as not possible to amplify the output from one of these cells because of their low internal resistance. However, it can be done very satisfactorily with a Tinsley d.c. amplifier since this is of the galvanometer input type, particularly if the output of the cell only varies slowly as in the present case. A magnetic amplifier would also be suitable. A possible disadvantage of the Tinsley amplifier is its high input resistance—of the order of 2000 Ω. It is generally stated that selenium barrier layer cells do not show a linear current/light relationship if the resistance in the external circuit exceeds 50–100 Ω. However, the large area cell used here responds linearly within the limits of our detection when combined with a Tinsley d.c. amplifier having an input resistance of 2300 Ω.

DESCRIPTION OF APPARATUS

The apparatus embodying these features is shown in Fig. 1. The paper, soaked in the immersion fluid, is carried upon a thin glass plate which is in turn supported by the framework of the trolley. The trolley runs on rails fixed on the top of the wooden stand below which is supported the light source. The trolley carries along one edge a strip of toothed rack which engages with a pinion of a synchronous clock motor which is fixed to the wooden base. The rack disengages on lifting the trolley, which can therefore be placed at any convenient position along the length of the track. The trolley is driven at the rate of 1 in./min. Since the chart of the recording milliameter is also driven by a synchronous motor, paper and chart always remain 'in step'. The slit, at the level of the top of the wooden base, has adjustable jaws and its effective length may be varied by laying on top of it brass masks with openings of various sizes between 5 and 25 mm. The light source is an 18 w, 6 v car-headlamp bulb with straight filament. This is mounted below the baseboard with the filament parallel to the slit and with the glass bulb almost touching the heat filter, which is carried in a frame attached below the slit carrier. Since it is important that the light should remain of constant intensity, it was found necessary to use a large-capacity battery as a current source. Constant-voltage transformers are useful if the mains have small fluctuations. The photocell, measuring $1\frac{1}{2} \times 2$ in., is mounted in a shallow frame carried on a short pillar. Its height can be adjusted so that whatever the thickness of the glass plate, etc., the sensitive surface is only 2–3 mm. above the filter-paper strip. A hinge is provided so that the cell can be swung out of the way while the trolley and its burden are being manipulated. The light filters are laid loosely on the slit. The whole of the space above the baseboard is enclosed by a light-tight box with an easily removable lid.

The d.c. amplifier and recording milliameter used have been those which are normally part of the Tinsley polarographic equipment. The polarograph control box has also been used to provide sensitivity control and zero-setting facilities.



Section through optical system

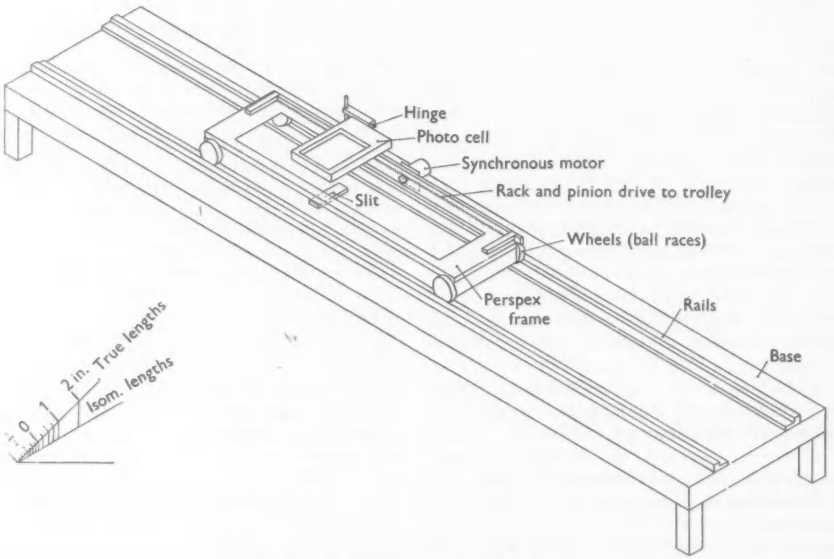


Fig. 1. Isometric projection of scanner.

To enable the recordings to be analysed, each is enlarged approximately $2\frac{1}{2}$ diameters by means of an episcopic projector. The recording, covered by a thin sheet of glass, is supported on a table in the light box where it is illuminated by four 60 w bulbs placed at the corners of the box. The lens, which is a Dallmeyer F 2·9, 8 in. focal length, focuses an enlarged image at the top surface of a sheet of plate glass covering a hole cut out of a table beneath which the light box is placed. A sheet of tracing paper is placed on this glass and the image followed round in pencil. Black curtains assist the procedure.

Preparation of strips

In experiments using serum or plasma, strips of no. 1 Whatman paper of area 38 × 5 cm. were used. For electrophoresis, Perspex tanks as described by Flynn & de Mayo (1951) were used. In the majority of experiments, approximately 5 μl. of serum and plasma were used on each strip. A veronal buffer of pH 8·6 and ionic strength 0·05 was usually employed, and a current strength of about 0·1 mA/cm. width of paper. A current-regulator was incorporated in the circuit in order to maintain a constant current through-

out the duration of the electrophoresis, which was usually carried out overnight for a period of about 16 hr. The strips were then dried in an oven at 100–110° and then stained.

It was found that the most convenient dye was azocarmine B, used according to the technique of Plückthun & Götting (1951). After being washed, the protein components appeared as red bands on a completely white background. The use of 'naphthalene black 12B.200' (Flynn & de Mayo, 1951) was also investigated. The chief disadvantage of this dye is that even after very protracted washing the background is still distinctly blue. When strips dyed with naphthalene black are used for direct photometry, the background colour introduces complications which are absent with azocarmine (cf. Fig. 9). Bromophenol blue, which is a satisfactory dye with the elution technique, is unsuitable for direct photometry because of its liability to change colour on exposure to the atmosphere.

After the final wash, the dyed strips were again dried in an oven at 100–110° and were then ready for impregnation with the immersion fluid. The immersion fluid used throughout these experiments was a mixture of equal volumes of α-bromonaphthalene and liquid paraffin (sp.gr. 0·880–0·895). A mixture of these two liquids was first suggested by Grassmann *et al.* (1951) and was found to be effective in reducing scatter, and has the added advantage of having no deleterious effect on Perspex. The dried paper strips are immersed in the mixture in a dish or beaker which is then transferred to an empty vacuum desiccator and evacuated with a water pump for about 20 min. A number of strips may be treated in this way simultaneously and if not required immediately for photometry should be left soaking in the liquid.

For photometry the strip is removed from the immersion fluid, drained momentarily, and laid flat on a thin glass plate (7·5 × 27 cm.). A strip of Cellophane is then placed over the whole paper strip and firmly pressed into close contact so as to exclude air trapped between the filter paper and the glass and between the filter paper and the Cellophane. The strip can now be transferred to the trolley and a recording made.

CALIBRATION

A first essential in calibrating the instrument is to ensure that the chart reads linearly in terms of light falling upon the photocell. In recording milliameters working on the Murday system, such as that supplied with the Tinsley Polarograph, the chart is not evenly divided. However, it is substantially linear between 10 and 90 divisions (Fig. 2), and in these experiments has usually been used between 20 and 80 divisions. To check the linearity of the photocell-amplifier-recorder combination, an Ilford neutral wedge was placed on the trolley and a recording of the light transmitted was made as it moved across the slit. The routine which was followed in this and all other recordings was the following. With the driving motor stopped, the wedge and trolley are positioned so that the slit image is in a clear portion of the wedge and the absorbing part of the wedge or paper will be carried over the slit when the driving motor is started. It is necessary to have the light on for these adjustments and in any event it is preferable to have the light on continuously, since this ensures a constant voltage from the battery and that the photocell has reached a steady state. Normally, the photocell is disconnected from the amplifier except during a recording. When the adjustments are complete, the light-proof lid is replaced, the recorder chart started and the recorder pen adjusted to division 20 by means of the zero-setting control. The photocell is now switched into the amplifier and the light intensity adjusted by means of a series resistance until the pen is at 80. Initially this may require adjustment of the sensitivity of the amplifier. Usually it has been found to be satisfactory to use this at the 1 μA range, that is, so that a 1 μA input from the photocell gives a full-scale deflexion. The trolley motor is now switched on and as the wedge or paper is traversed across the slit the milliameter records the decrease of photocell

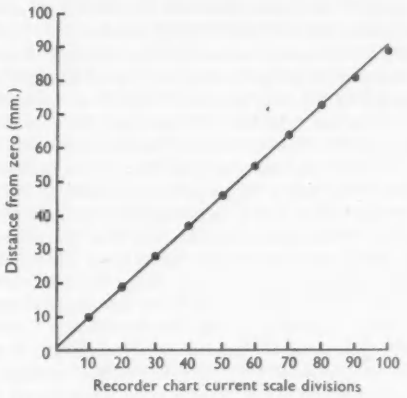


Fig. 2. Graph showing essential linearity of recorder chart current scale divisions over the normal working range of 20–80 scale divisions.

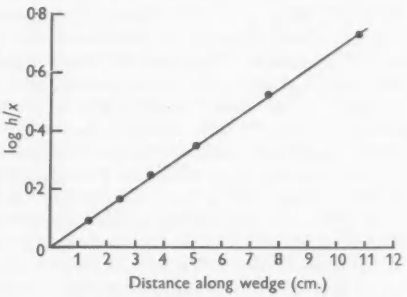


Fig. 3. Log h/x plotted against distance along wedge for a recording of light transmitted during transit of an optical wedge. h = excursion of milliameter with full light intensity; x = excursion with absorbing medium.

current. Since the optical density of the neutral wedge varies linearly with length, if 'h' is the excursion of the milliameter with full light intensity and 'x' is the excursion with an absorbing medium in the light path, a plot of log h/x should be linear for a recording from the wedge provided the photocell-amplifier-recorder combination is linear. Fig. 3 shows that the response of the combination is substantially linear.

It appears to have been generally assumed by other workers (e.g. Grassmann *et al.* 1951; Latner, Braithwaite & Nunn, 1952) that the light absorption by dyed protein in an immersed filter paper obeyed Beer's law. However, the system is optically very complex, both because of the residual scattering of light and also because the protein-dye complex is not uniformly distributed, being in the form of irregular aggregates of dyed protein of different sizes distributed non-uniformly within the depth of the paper. It seemed essential, therefore, to test experimentally the validity of Beer's law.

Serial dilutions in saline of a solution of dried, citrated human plasma were prepared. Filter paper disks (12·5 cm.) were saturated with these dilutions by placing a disk in the bottom of a Petri dish and quickly pouring on the diluted plasma. It is essential that the dish and the paper should be dry initially and that the paper be completely wetted at once, otherwise non-uniform impregnation with protein results. After soaking for a few minutes, the papers were dried and dyed in the usual way. From the central, uniform portion of each disk an area 6×5 cm. was cut out and divided into three strips, 2×5 cm., numbered 1, 2 and 3. Nos. 1 and 3 were placed in test tubes and the dye was eluted from them by treatment with 0·1 N-NaOH and measured in a photoelectric colorimeter, care being taken to ensure that the dilution of the dye was sufficient to bring the readings into the range where Beer's law was obeyed. The concentration was expressed in arbitrary units based on the colorimeter reading and the dilution. Strip no. 2 was soaked in immersion fluid and, in close juxtaposition with similar strips from other plasma dilutions, was laid upon a glass plate to form a graded series of increasing depth of colour—an 'optical wedge' of dyed protein. At the beginning and end of this 'wedge', strips of immersed but otherwise untreated paper were placed to provide a base-line. The whole was then placed on the trolley and a recording made. A typical result is shown in Fig. 4. The sloping tops of the steps are not due to non-uniform dyeing of the strips but to the effect discussed earlier of the surrounding dyestuff upon the light absorption, and possibly also to pen-friction in the recorder. The value of the current at the centre of the strip can be taken as the correct value of 'x' for each dye concentration. When log h/x is plotted against concentration of dye in arbitrary units, a straight line should result if Beer's law is being obeyed. As can be seen from Fig. 5 this is very far from being true. There are appreciable deviations at quite low dye concentrations.

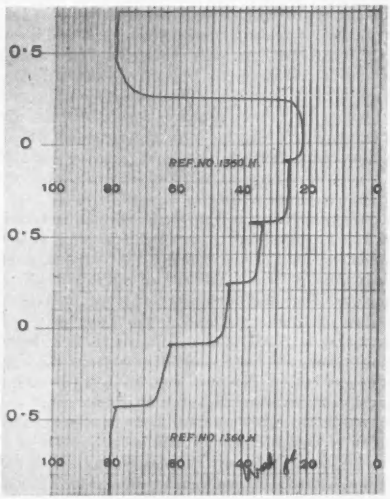


Fig. 4. Photograph of actual recording of azocarmine 'step-wedge'. Milliameter adjusted to 80 scale divisions for transmission through undyed paper. 20 scale divisions correspond to complete extinction. The wedge contained five steps made by serial dilution of 1, 1/2, 1/4, 1/8, 1/16.

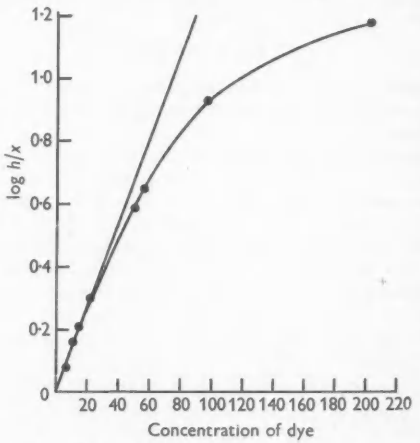


Fig. 5. Log h/x for the recorded current plotted against the concentration of dye (in arbitrary units) actually on the paper, as determined by elution. The straight line is calculated from Beer's law.

In attempts to find a suitable law to express the relation between current and dye concentration, it was found empirically that the relation between the decrease in photocell current 'y' and the dye concentration in arbitrary units is well represented over the working range by a rectangular hyperbola of the form:

$$y = \frac{ac}{b+c},$$

where 'c' = concentration and 'a' and 'b' are constants. This can be more readily seen by taking reciprocals, when

$$1/y = \frac{b+c}{ac} = \frac{b}{a}\frac{1}{c} + \frac{1}{a}.$$

Thus, when $1/y$ is plotted against $1/c$ (Fig. 6), a straight line results, of which the intercept on the ordinate is $1/a$ and the slope b/a. From this plot, the constants for the best fit of a set of experimental points are readily deduced. It must be emphasized that the hyperbolic relation no longer holds under conditions of extremely dense staining.

Later it was found to be unnecessary to impregnate the paper with protein before applying the dye. Simple dipping of filter paper disks in serial dilutions of dye solution followed by drying was found to give identical calibration curves. Nor in the case of azocarmine nor naphthalene black, two dyes with quite different absorption spectra, was the curve altered by changing the dye, provided the recording was made with a light filter passing only the major absorption peak of the dye used. Unsuitable filters or the absence of a filter gave curves falling below that in Fig. 6. Fig. 6 includes points obtained from wedges of protein dyed with azocarmine B, with azocarmine B without protein, and with naphthalene black without protein.

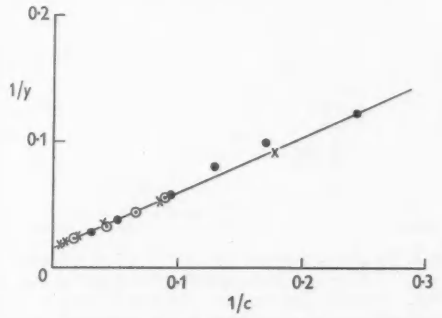


Fig. 6. Reciprocal of the decrease of photocell current ($1/y$) plotted against the reciprocal of the dye concentration ($1/c$). ● = naphthalene black; ○ = azocarmine B, × = azocarmine B + protein. Ilford filters 608 (napthalene black) and 624 (azocarmine B).

It is thus obvious that assuming the applicability of Beer's law will lead to grossly erroneous results, the errors being greatest when comparing a high with a low concentration.

The pronounced curvature of the calibration curve has further important implications for the slit dimensions and the amount of protein placed upon the paper. Since the decrease in photocell current for large concentrations of dye is small in proportion, the presence of small amounts of extraneous radiation will cause a large error when calculated in terms of protein. Use of a wide slit for scanning bands whose edges rise sharply is therefore liable to introduce serious 'optical dilution', resulting in a lowering in the height of the recorded peak. It has been found necessary to have the slit less than 1 mm. wide to avoid this effect with the strips used in these experiments.

Similarly, it at first appeared best to limit the protein band to less than the total width of the paper and to scan with a slit whose length was sufficient to project beyond the bands and ensure complete integration of all the colour. However, the diluting effect of the undyed paper at the ends of the slit reduced the albumin peak seriously, and it was found to be necessary to use a slit short enough to take in only the central uniform portion of the bands.

Increasing the amount of protein too much results, not only in poor strips due to 'overloading', but also brings the albumin peak into the flat portion of the calibration curve and makes the record insensitive to variations in the amount of albumin. This last effect becomes important at lower concentrations than does 'overloading' and for best records, strips should be run which would be judged rather too pale for comfortable examination by eye.

ANALYSIS OF CURVES

When a paper strip carrying bands of dyed protein resulting from the electrophoresis of, say, normal human serum is run through the recorder, there results an 'electrophoresis diagram' closely resembling that obtained with the classical Tiselius equipment. Some typical results are shown in Fig. 7. The chief differences are (1) that the albumin peak is considerably depressed owing to the flattening out of the calibration curve at high dye concentrations and (2) that here each peak represents the total protein making up that component, whereas in the classical diagram it represents the concentration change due to adding that component to a mixture of other components. The total area under a peak is a function of the total dye in that part of the paper, which in turn represents the total amount of protein in the particular serum protein component. The chief question that arises in attempting to assign a given area under the curve to

a particular protein is, what is the dividing line between it and its nearest neighbours. Three chief methods of deciding upon the separation of the components have been used in analysing the classical Tiselius diagrams. The oldest is simply to drop perpendiculars from the troughs of the curve to the base-line and to regard the area enclosed between each adjacent pair of perpendiculars as a measure of the amount of that component. Alternatively, if the peaks are symmetrical in outline, the inner edges of the albumin and γ-globulin peaks can be obtained by reflecting the outer edges about the perpendicular from the peak (Svedberg & Pedersen, 1940). Subtraction of these inner 'tails' from the total curve gives a residual curve for the remaining components and the process can be repeated with this. A third method depends upon the assumption that, in the absence of distorting influences, the shape of the peak is due to the diffusion of the boundary and therefore should resemble a normal Gaussian error curve. It is possible to fit such curves to the diagram by the methods of Labhart (1947) and of Wiedemann (1947). The results obtained by these more elaborate methods do not differ very much from those obtained by simple perpendicular dropping, and in any case they are not applicable here, since the peaks are not symmetrical and do not fit normal error curves. Perpendicular dropping was, therefore, used in this work and has the advantage that it is the simplest and most consistent of the methods for drawing arbitrary lines of demarcation and is, in fact, the method which results when elution methods of analysing the paper strips are used. Its chief disadvantage is that it tends to exaggerate a minor component which occurs as a shoulder on the side of a major component, e.g. the α_1-globulin in most sera.

As the calibration curve is not linear, the area under a peak is not necessarily directly proportional to the total amount of dye, i.e. to the total amount of protein in the particular components. However, the initial part of the curve (up to one-fourth of the total range) is effectively linear, and for peaks which fall below this level the area of the peak can be taken as an estimate of the amount of the component present within the experimental error. With most strips, certainly those dyed with azocarmine B, it is easy, and indeed desirable for other reasons, to arrange the total amount of protein so that only the albumin peak rises above the straight portion of the calibration curve. The remaining components can therefore be estimated by direct planimetry.

A simple graphical method was developed for correcting the albumin peak so that its area becomes

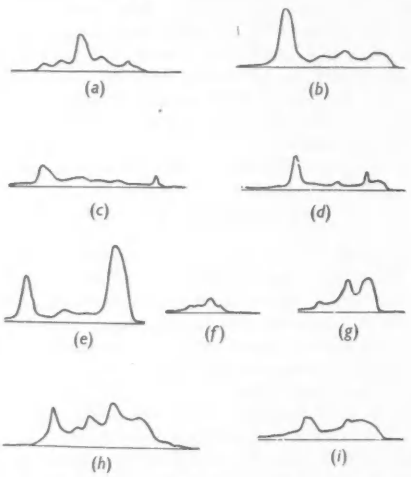


Fig. 7. Tracings of illustrative recordings: (*a*) human nephrotic serum; (*b*) normal human serum; (*c*) foetal goat plasma; (*d*) maternal goat plasma; (*e*) human lymphatic leukaemia serum; (*f*) human milk in phthalate buffer pH 6·0; (*g*) goat colostrum without caseinogen; (*h*) partially purified hexokinase preparation; (*i*) goat colostrum. With the exception of (*f*) all samples run in barbitone buffer pH 8·6. The fastest moving component appears on the left of the tracing.

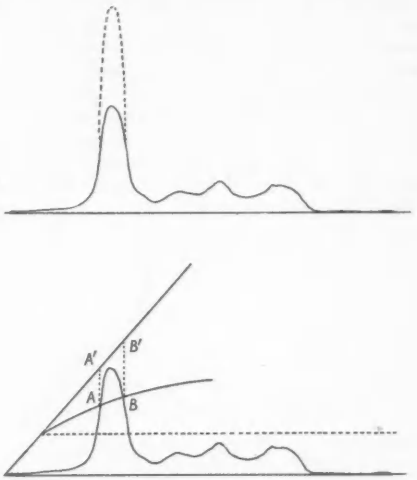


Fig. 8. Diagrams showing method of using calibration curve for correcting high peaks for deviation from Beer's law. Points of interception of a high peak with the calibration curve (*A* and *B*) are replotted vertically on the linear projection of the initial part of the calibration curve (lower diagram). By lateral movement of the peak this process may be repeated and the corrected peak constructed as shown by the dotted curve in the upper diagram.

directly proportional to the amount of albumin, which can therefore also be measured by planimetry. The calibration curve is redrawn so that its vertical scale is enlarged by the same factor as are the tracings obtained by projection from the original records. The concentration scale is quite arbitrary. The initial straight portion of this curve is projected as shown in Fig. 8. The tracing of a record is then placed over the calibration curve and projected straight portion, and adjusted so that its base-line coincides with the base of the calibration curve. In most instances the albumin curve will cut the calibration curve at two points, say A and B. If the decrease in photocell current due to these two concentrations of dye had been directly proportional to the dye concentration, the points would have occurred at the positions vertically above these and upon the projected straight line at A' and B', respectively. By shifting the tracing sideways, keeping its base-line co-incident with the base of the calibration curve, another pair of 'proportional points' can be found, and so on until a sufficient number has been plotted to allow a freehand curve to be drawn connecting them. This will merge into the original albumin curve at the height at which the calibration curve diverges from its initial linear portion. If the calibration curve and projected line are drawn on graph paper, finding the points vertically above the intersections is very simple and the whole process of 'correcting' the albumin peak by this method only occupies 3 or 4 min. The corrected albumin peak thus obtained, since the height of every point on it is directly proportional to the dye concentration at that point, has an area directly proportional to the total amount of the albumin, and is ready for planimetry.

This last operation is carried out by placing the tracing on a flat surface beneath a thin sheet ($\frac{1}{16}$ in. or less) of Perspex, which provides an excellent surface for the planimeter to work on and holds the tracing flat and prevents it from moving about.

It is because of the necessity of correcting the record that naphthalene black is an unsuitable dye. With azocarmine B, the background is completely undyed and only the albumin peak reaches into the region of substantial curvature of the calibration curve. With naphthalene black, on the other hand, even prolonged washing does not remove the dye from the paper, and the electrophoresis diagram is therefore superimposed upon a considerable continuous background which raises all of the peaks into the region of curvature. A tedious correction of the whole curve by the method described above is then necessary. Fig. 9*b* shows a record from a naphthalene-black dyed strip, with the corrections, for comparison with the azocarmine-B dyed strip in Fig. 9*a*. Strips of undyed but immersed papers at each end of the naphthalene-black strip are included in order to provide a base-line from which the corrections are made, and the final areas under the peaks are from this base-line.

REPRODUCIBILITY OF RESULTS

It was found that the reproducibility of the recordings obtained from repeated runs on a given strip was good. That is, the actual curves recorded are, for all practical purposes, superimposable. However, in considering the quantitative evaluation of components in a given serum, there are several stages in the procedures for recording and analysing the curves where errors might be introduced. In

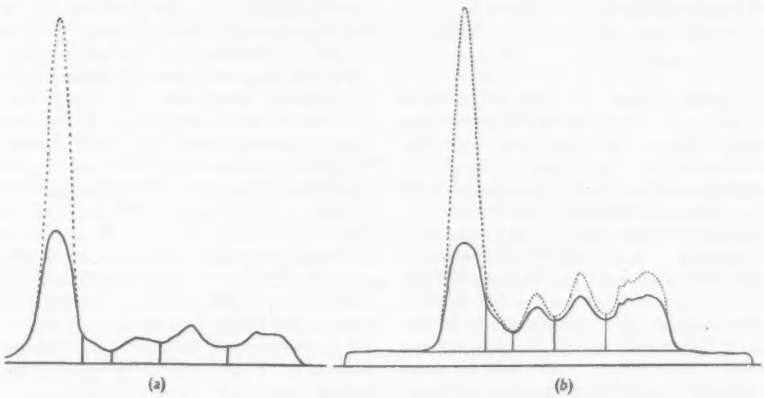


Fig. 9. Comparison of original (full line) and corrected (dotted line) recordings from serum stained with azocarmine B (*a*) and with naphthalene black (*b*). The low plateau in (*b*) represents cellulose-adsorbed dye not removed by the washing process.

Table 1. *Analysis of variance for thirty-two replicate recordings on the same sample of serum*

(Eight separate strips were prepared and each was run four times through the photometer.)

Fraction	Mean % of total protein	Components of variance: Between strips σ_B^2	Components of variance: Between runs σ_W^2	Total variance	S.D.
Albumin	60·5	3·02	2·50	5·52	2·35
α_1-Globulin	3·9	0·15	0·57	0·72	0·85
α_2-Globulin	8·7	0·89	2·18	3·07	1·75
β-Globulin	10·2	0·82	0·30	1·12	1·06
γ-Globulin	16·7	1·09	0·75	1·84	1·36
	100·0				

a detailed examination of the procedure from this point of view, it was found that the variation in results largely arose from the inherent difficulties of that part of the analysis involving the delimitation of the fractions on the diagram. Replication experiments indicated that the standard deviation of the percentage of any given component was of the order of 1–2 % of the total protein. A typical result is shown in Table 1. In this experiment eight replicate strips were run on a single sample of serum from one subject. No attempt was made to apply exactly the same quantity of serum to each strip. The strips were stained with azocarmine, washed and dried in the usual way. Four independent recordings were then made from each strip, the strip being re-immersed between each recording. The thirty-two recordings thus obtained were then analysed independently and the variance of the percentage of each fraction was divided into two components. One component, σ_B^2, represents the variation between individual strips and σ_W^2 that between replicate recordings on the same strip. These two components are of the same order of magnitude for each fraction.

DISCUSSION

This investigation has shown that, with attention to certain important points, it is possible to analyse human serum by paper electrophoresis with the same order of error as that obtained with the classical Tiselius apparatus, but using apparatus of about one-tenth the cost. Also, at least ten times as many analyses can be performed in the same time. The question therefore arises as to how well the results of the older established and more elaborate apparatus are reproduced by the newer and simpler techniques. In attempting to discuss this question it is important that certain points should be kept clearly in mind.

The first point to be remembered is the arbitrary nature of the fractions separated by the classical procedure. It is well known that although, in general, electrophoresis of human serum under standard conditions produces only five components (albumin, α_1-, α_2-, β- and γ-globulins), many more proteins than this are actually known to be present. Thus, Cohn and his collaborators (e.g. Cohn, Oncley, Strong, Hughes & Armstrong, 1944; Cohn *et al.* 1946) have separated more than a dozen fractions differing clearly in their physico-chemical properties, a large number of enzymes are known to be present in serum, and most sera contain a host of antibodies of one sort or another. Many of the peaks on the classical electrophoresis diagram must therefore represent mixtures of proteins and this has been demonstrated for the α- and β-globulins by Cohn's fractionations. Comparison of electrophoresis with ultracentrifugation and salt precipitation serves only to emphasize the arbitrary nature of any of the fractions produced by whatsoever method from so complex a mixture as serum.

Considerations such as these suggest that the regions below the troughs in the classical diagrams also represent mixtures and may contain components other than those represented by the adjacent peaks. A recent experiment by Kunkel & Tiselius (1951), in which serum was separated electrophoretically in filter paper in two directions at right angles, supports this.

In analysing schlieren diagrams quantitatively, it is generally assumed that the regions below the troughs represent mixtures of the components forming the adjacent peaks caused by spreading of the peaks owing to diffusion and errors in the separation procedure, and contain no other components. Indeed, in most instances, no other assumption would be possible in the absence of other data as to the true number of protein components present, and even with such data there would be no obvious method for assigning particular areas to particular components. It is obvious that a large number of different combinations of components could give rise to the same electrophoretic diagram.

These facts are generally admitted by those working on the electrophoresis of proteins. However, there is no doubt that, in practice, they are

largely ignored in interpreting electrophoresis diagrams or in arguments concerning the relationship of the results of electrophoresis to other evidence about the properties of proteins and protein mixtures. Thus, it is frequently reported that, in a partially purified protein preparation, 'the main component is accompanied by $x\%$ of another protein of lower mobility', the implication being that each is a single entity. Often this assumption is subsequently found to be justified, but not invariably so.

It is often assumed that the classical electrophoresis diagram provides an absolute standard to which other methods of separation should be referred. However, in view of the facts mentioned above, there could be no *a priori* expectation that electrophoresis in filter paper with subsequent dyeing should produce results identical with those obtained in the classical apparatus. The fact that the picture obtained for normal sera separated in filter paper and subsequently dyed resembles very closely that generally obtained by the older technique can be regarded as nothing more than an interesting and useful coincidence.

Whether the two techniques will always give qualitatively similar pictures remains to be determined. It is certain already that there are quantitative differences. In discussing the reasons for these differences and the criteria to be used in assessing the paper electrophoresis diagrams, a second point must be borne in mind, namely, the arbitrary nature of the quantitative interpretation of both the classical and paper electrophoresis diagrams.

Apart from the assumption that only the major components are present and that it is possible to fix lines of demarcation between these, two other major assumptions are made in converting the areas under a classical electrophoresis curve into their equivalents in protein. The first is that the change in refractive index at the boundary between components is caused solely by changes in the concentration of protein, and the second is that the specific refractive increments of all components are the same, or alternatively that the specific refractive increments of all globulin components are equal to that of γ-globulin, and that these remain the same from one serum to another, in health and in disease, and are independent of the admixture of varying quantities of other components.

Neither of these assumptions is true. The presence of the so-called δ and ϵ anomalies makes quite clear that changes in the buffer and salt concentrations do occur. It is generally assumed that no such anomalies are associated with the protein components, other than the β-peak on the descending limb where the occurrence of the so-called β-anomaly has usually caused investigators to confine their quantitative analyses to the ascending limb. However, particularly in the albumin and γ-globulin components where there is a junction between buffer and buffer + protein, the occurrence of Donnan equilibria and of changes in pH and electrolytic conductance would make it inevitable that there are substantial changes in the concentration of buffer and salts, i.e. that these peaks are due partly to protein and partly to 'anomalies'. This has recently been confirmed for the albumin peak by Svensson (1943), Perlman & Kaufmann (1945) and by Koling, Perring & Hogness (1946). Svensson's results suggest that the proportion of albumin which, for an average normal serum, is usually measured as approximately 60 %, should be approximately 56 % when allowance is made for the accompanying anomaly. Although investigations have not yet been made, there is no reason to assume that similar effects are absent at the other peaks.

Little information is available on the variation of the refractive index increment of the different components, or of how this is affected by the environment. However, it is unlikely that lipoproteins, some of which are known to contain up to 75 % lipid, would have the same value for this constant, as, say, albumin. Moreover, proteins other than those present in serum are known to vary considerably in this respect (e.g. Putzeys & Brosteaux, 1936).

Nothing that has been said above should be interpreted to mean that the assumptions are not useful or even that other assumptions are possible. They have only been enumerated to demonstrate that the usual quantitative interpretations of the classical electrophoresis diagram can have no claim to absoluteness and indeed represent an entirely empirical method of analysis giving strictly comparable results only when carried out under carefully standardized conditions. As this is almost never done, most of the data in the literature which have been produced by this apparatus can only be approximately compared.

Quantitative evaluation of dyed-paper electrophoretic diagrams involve assumptions similar to those necessary for the classical diagrams. Again it must be assumed that only the major components are present and that there is a clear-cut line of demarcation between them. In addition, it has to be assumed that only protein takes up the dye, and, if the results are to be reported in terms of per cent protein, that all components take up the dye to the same degree. Alternatives to this will be discussed later. In addition, a further assumption has until now been made by most workers in this field, namely, that the relation between the concentration of the dye and the photocell current is that described by Beer's law. This last assumption has been shown

in this paper to be invalid and to lead to serious under-estimation of the amount of dye in the albumin band. Using an elution method, and bromophenol blue as their dye, Cremer & Tiselius (1950) and Kunkel & Tiselius (1951) found it necessary to multiply the globulin components by factors of about 1·6 to bring their results into line with those of the classical method. The question arises as to whether it is advantageous to apply such correction factors so as to bring the paper results into line with the classical values, or alternatively, whether calibration factors based on nitrogen, dry weight, or on some other property of the fractions should be used.

It appears to the authors that correction factors are neither necessary nor desirable. Even though it may be possible to determine correction factors for each peak for converting paper values into classical values, little is gained, since the classical values are not strictly comparable among themselves; nor is there any guarantee that these factors will remain the same from one serum sample to another or from one disease to another. In any event, the lack of any fundamental basis for the classical values suggests that it would be preferable to build up a new set of data based entirely upon the paper technique.

The alternative proposal that the amount of dye absorbed by any component should be calibrated in terms of nitrogen or dry matter, etc., is open to exactly the same objections, namely, the difficulty of separating the components in order to carry out the calibrations and the uncertainty that the calibrations will be unvarying from one protein mixture to another or from one disease to another. Indeed, it is well known that the proportion of nitrogen in the various serum components varies considerably.

It is therefore concluded that expression of the analyses in terms of the total amount of dye adsorbed by components separated and dyed under standard conditions is at present the most satisfactory basis for measurement. There is nothing in this to preclude the investigation of changes in the composition of sera and other protein mixtures under various conditions, whether these changes are due to variation in the actual amounts of the components or in their relative ability to adsorb dye. Indeed, whatever the basis of measurement, there is little to distinguish these two factors unless more than one criterion is applied simultaneously.

SUMMARY

1. An apparatus is described for the continuous direct photometry of dyed materials in filter paper, which has been successfully employed for the estimation of proteins separated by electrophoresis.

2. The applicability of Beer's law to the absorption of light by dyed protein in an immersed filter paper was tested experimentally. It was found that, outside of a very restricted range, large deviations occur.

3. A method of calibration is described by which the deviation from Beer's law may be estimated and compensated for.

4. The relation between the results of electrophoretic analysis of proteins in paper and in free solution are discussed.

We are indebted to the Central Research Fund of the University of London for a grant for the purchase of apparatus used in this work. F. Hassan participated during the tenure of an Egyptian Government Scholarship.

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CORRIGENDA

Relations between metabolism and the rate of turnover of sodium and potassium in guinea pig kidney-cortex slices

By R. Whittam and R. E. Davies

Volume 56 (1954)

p. 447, Table 2, heading of column 4:

for Aerobic with 2mM 2:4-dinitrophenol
read Aerobic with 0·2mM 2:4-dinitrophenol

p. 448, col. 2, line 10:

for 2mM *read* 0·2mM

p. 450, Table 5, description for 3rd line of figures:

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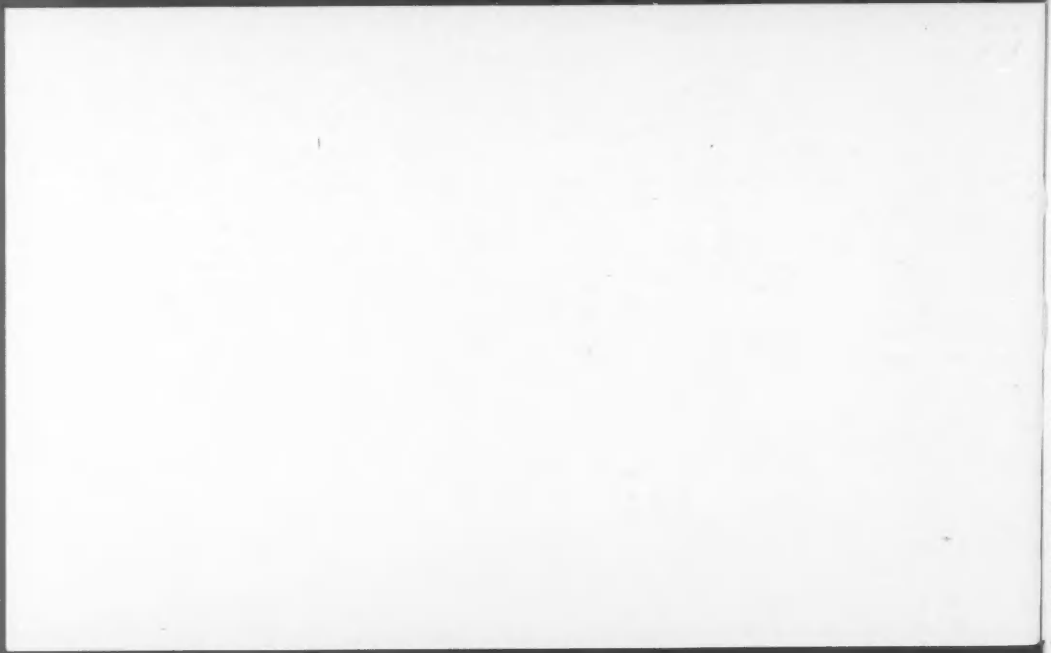

Relations between Metabolism and the Rate of Turnover of Sodium and Potassium in Guinea Pig Kidney-cortex Slices

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It is now known that sodium and potassium ions exchange rapidly between cells and body fluids, and that the observed concentration gradients are not due to impermeability of the cell membrane (see reviews by Krogh, 1946; Ussing, 1949). It is also known that the production and maintenance of physiological concentration gradients of sodium and potassium in isolated kidney-cortex slices require respiratory energy (Krebs, Eggleston & Terner, 1951; Mudge, 1951*a*, *b*; Aebi, 1953; Whittam & Davies, 1953*b*).

In order to investigate the relation between the to-and-fro movements of sodium and potassium and the metabolism of this tissue, the turnover-rates of these ions have been measured in several steady-state conditions.

A preliminary account of this work has been presented to the Biochemical Society (Whittam & Davies, 1953*a*).

EXPERIMENTAL

Incubation of tissue. Guinea pig kidney-cortex slices were prepared and incubated as described by Whittam & Davies (1953*b*). In some experiments the tissue was placed directly into 2 ml. of bicarbonate saline, containing either $^{24}Na^+$ or $^{42}K^+$, in 15 ml. stoppered vessels, but it was usually pre-incubated for about 35 min. and then transferred to the radioactive medium.

Measurements of Q_{O_2} (μl./mg. dry wt./hr.) were made in Warburg manometers in the usual way.

Estimation of sodium and potassium. The tissue (approx. 150 mg.) was dissolved in 16 N-HNO_3 and diluted to 5 ml. with water (for details see Whittam & Davies, 1953*b*). Samples (1·0 or 2·0 ml.) were diluted to 10 ml. with either 0·0382% (w/v) KCl solution or 0·84% (w/v) NaCl solution. These solutions and samples of the medium were used for determination of Na^+ or K^+, respectively, with a flame photometer (Domingo & Klyne, 1949).

This procedure measures the total sodium and potassium, but the symbols Na^+ and K^+ have been used throughout to include any undissociated or un-ionized material which may be present.

Handling of $^{24}Na^+$ *and* $^{42}K^+$. Spectroscopically pure $^{24}Na_2CO_3$ and $^{42}K_2CO_3$ were supplied by the Atomic Energy Research Establishment, Harwell, and neutralized with HCl; enough was added to give samples of the medium with about 6000 counts/min. The tracer amounts of $^{24}Na^+$ and $^{42}K^+$ added in this way did not appreciably change the concentrations of Na^+ or K^+ in the medium. The radio-activity of medium and tissue was measured in an M. 6 liquid counter (Veall, 1948) and corrections were made for isotope decay, dead time and background counts. $^{24}Na^+$ was measured in solutions prepared by diluting 0·05 ml. of medium and 2·0 ml. of the acid digest of the tissue to 10 ml. with 0·9% (w/v) NaCl solution. $^{42}K^+$ was measured in solutions prepared by diluting 1·0 ml. of medium and 2·0 ml. of the acid digest of the tissue to 10 ml. with 0·1 N-KCl solution.

RESULTS

Exchange of the sodium of kidney-cortex slices on incubation in vitro

Non-steady-state exchanges. Table 1 shows the effect of various periods of aerobic and anaerobic incubation at 37° on the amount of exchange between sodium in the tissue and medium. The rate of exchange was higher aerobically, when the sodium content was lower, than anaerobically when the sodium content was higher. Under aerobic conditions there was nearly complete exchange (98%), and under anaerobic conditions 96% exchange in 2 hr. After 2 hr. aerobic and anaerobic incubation at 0°, there was an exchange of 92% of the tissue sodium. These results show that there was little, if any, non-exchangeable sodium in these kidney-cortex slices at either 0° or 37° in aerobic or anaerobic conditions.

Steady-state exchanges. After a preliminary incubation period of about 35 min., when the sodium content of the tissue had become approximately steady, the slices were placed in saline containing radioactive sodium. The uptake of this ion by the tissue during the following few minutes in aerobic conditions at 37° is shown in Fig. 1, and in anaerobic conditions at 37° and 0° in Fig. 2. There was in each case a rapid initial increase in the percentage exchange, but after about 1 min. the rate of increase became much slower.

The formula of Krebs *et al.* (1951) for the calculation of the steady-state turnover of material in a two-component system is applicable to the measurement of turnover-rates of the intracellular sodium only when a correction of the experimental measurements can be made for the amount of extracellular sodium present in the tissue. Since this amount was not known precisely, an alternative approach has been made to interpret the results.

Table 1. *Changes in the distribution of $^{24}Na^+$ between guinea pig kidney-cortex slices and medium during incubation at 37°*

(Tissue (0·1–0·2 g.) incubated in 2·0 ml. of bicarbonate saline containing $^{24}Na^+$ and 10 mM α-oxoglutarate; pH 7·4, gas phase 5% CO_2 and either 95% O_2 or 95% N_2.)

Experimental conditions	Period of incubation (min.)	Na^+ content of tissue after incubation* (μmoles/g. tissue)	Specific activity of Na^+ in tissue (counts/min./μmole Na^+). After incubation	Specific activity of Na^+ in medium (counts/min./μmole Na^+) Before incubation	Specific activity of Na^+ in medium (counts/min./μmole Na^+) After incubation	Ratio: (specific activity of Na^+ in tissue/specific activity of Na^+ in medium) × 100 (% exchange)
Aerobic	0·27	78·0	776	1365	1255	61·8
	1·0	88·2	1050	1365	1280	82·1
	4·9	100	1080	1365	1140	94·8
	5·8	90·1	1160	1365	1235	94·0
	6·4	91·8	1220	1365	1250	97·7
	7·6	104	1025	1185	1170	87·6
	15·2	97·9	1160	1185	1215	95·5
	30·3	99·2	1149	1185	1136	101
	60·3	102	1095	1185	1153	95·1
	120·0	98·5	1100	1185	1130	97·4
	120·5	98·4	1208	1365	1230	98·4
Anaerobic	0·27	77·0	763	1325	1385	55·1
	0·5	82·5	992	1325	1285	77·2
	1·8	95·8	1120	1325	1300	86·3
	4·5	104	974	1325	1295	75·2
	7·7	114	1135	1261	1328	85·5
	15·2	125	951	1261	1207	78·8
	30·0	120	1106	1261	1225	90·4
	60·2	136	1095	1261	1237	88·6
	120·0	122	1176	1261	1218	96·7
	120·1	131	1250	1325	1300	96·1

* Sodium content of tissue before incubation = 58·9 μmoles/g. tissue.

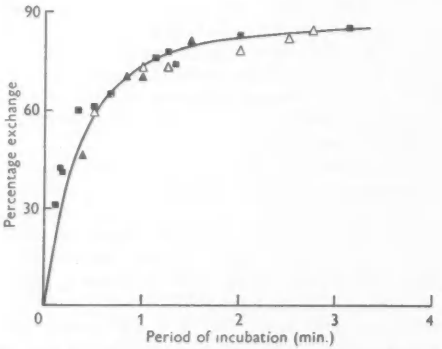

Fig. 1. The uptake of radioactive sodium by guinea pig kidney-cortex slices in steady-states at 37°. About 0·2 g. tissue incubated in 2·0 ml. of bicarbonate saline; gas phase, 5% CO_2 and 95% O_2; pH 7·4. After approx. 35 min. incubation the tissue was transferred to a similar saline solution containing ^{24}Na. ■, saline with 10 mM α-oxoglutarate; ▲, saline without additions; △, saline containing 10 mM α-oxoglutarate and 0·2 mM 2:4-dinitrophenol. The curve represents the function

$$P = 76[1 - \exp(-2{\cdot}65t)] + 24[1 - \exp(-0{\cdot}151t)],$$

where P is the percentage exchange of tissue sodium and t is the period of incubation in minutes in the radioactive medium.

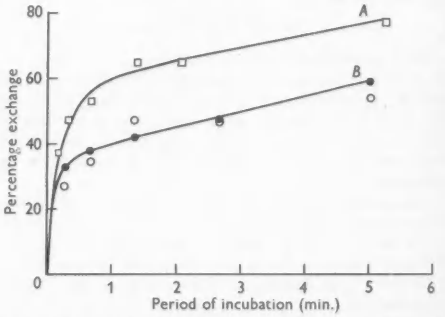

Fig. 2. The uptake of radioactive sodium by guinea pig kidney-cortex slices in steady-states at 37° and 0°. About 0·2 g. tissue was incubated in 2·0 ml. of bicarbonate saline containing 10 mM α-oxoglutarate at 37° (gas phase, 5% CO_2 and 95% N_2; pH 7·4) or at 0° (gas phase, 2·8% CO_2 and either 97·2% O_2 or 97·2% N_2; pH 7·4). After approx. 35 min. incubation the tissue was transferred to a similar saline solution containing ^{24}Na. □, anaerobic conditions at 37°; ○, aerobic conditions at 0°; ●, anaerobic conditions at 0°. Curve *A* represents the function

$$P = 55[1 - \exp(-4{\cdot}45t)] + 45[1 - \exp(-0{\cdot}134t)],$$

and curve *B* the function

$$P = 34[1 - \exp(-8{\cdot}28t)] + 66[1 - \exp(-0{\cdot}0954t)],$$

where P is the percentage exchange of tissue sodium and t is the period of incubation in minutes in the radioactive medium.

The experimentally determined points in Figs. 1 and 2 are in good agreement with the curves representing the empirical functions,

$$P = 76[1 - \exp(-2{\cdot}65t)] + 24[1 - \exp(-0{\cdot}151t)]$$

for aerobic conditions with or without substrate and in the presence of 2:4-dinitrophenol at 37°;

$$P = 55[1 - \exp(-4{\cdot}45t)] + 45[1 - \exp(-0{\cdot}134t)]$$

for anaerobic conditions at 37°; and

$$P = 34[1 - \exp(-8{\cdot}28t)] + 66[1 - \exp(-0{\cdot}0954t)]$$

for aerobic and anaerobic conditions at 0°, where P is the percentage exchange of the tissue sodium, and t is the period of incubation in minutes. The figures before the square brackets represent the percentages of tissue sodium exchanging with the rate constants given within the brackets.

This shows that there are at least two major fractions of the tissue sodium, which exchange according to first-order processes. However, both the relative sizes and the velocity constants of these components changed with the experimental conditions. Aerobically at 37°, 50% equilibration was reached in 16 sec. by the rapidly exchanging fraction of the tissue sodium, and in 4·6 min. by the slowly exchanging fraction (Table 2).

The amount of sodium in the rapidly exchanging fraction at 37° in the presence of oxygen was 76%, which is surprisingly high. This fraction consisted of sodium indistinguishable from that of true extra-

Table 2. *Steady-state turnover-rates of $^{24}Na^+$ in guinea pig kidney-cortex slices during incubation*

(Tissue (0·1–0·2 g.) incubated in 2·0 ml. of bicarbonate saline containing 10 mM α-oxoglutarate; pH 7·4; gas phase at 37°, 5% CO_2 and either 95% O_2 or 95% N_2; gas phase at 0°, 2·8% CO_2 and either 97·2% O_2 or 97·2% N_2. Pre-incubated for 35–40 min. and then placed for various times in similar saline containing $^{24}Na^+$. Data derived partly from graph of percentage exchange against time. For further details see text and Fig. 1.)

Temperature of incubation ...	37°				0°	
Experimental conditions ...	Aerobic	Aerobic without added substrate	Aerobic with 0·2 mM 2:4-dinitrophenol	Anaerobic	Aerobic	Anaerobic
Na^+ content of tissue after incubation* (μmoles/g. tissue)	99·5	108	124	114	113	
Amount of rapidly exchanging Na^+ in the tissue (%)	76	76	76	55	34	
Space of tissue occupied by rapidly exchanging part of Na^+ ('outer' space) (%)	48·4	52·3	60·2	40·0	24·5	
Na^+ content of part of tissue occupied by slowly exchanging Na^+ (μmoles/g. tissue contained in 'inner' space)	46·0	54·3	74·4	85·6	98·8	
Ratio: Na^+ content of part of tissue occupied by slowly exchanging Na^+/Na^+ concentration in medium	0·293	0·346	0·474	0·546	0·630	
Rate constants of component parts of tissue Na^+ (min.$^{-1}$):						
Fast	2·65	2·65	2·65	4·45	8·28	
Slow	0·151	0·151	0·151	0·134	0·0954	
Time for half-exchange of the components of tissue Na^+ (min.):						
Fast	0·26	0·26	0·26	0·16	0·084	
Slow	4·6	4·6	4·6	5·2	7·3	
Turnover-rates of slowly exchanging parts of tissue Na^+ (%/min.)	16·2	16·2	16·2	12·1	8·94	
Exchange-rates of slowly exchanging parts of tissue Na^+:						
(μmoles/min./g. whole tissue)	3·92	4·20	4·80	6·21	6·67	
(μmoles/min./g. tissue contained in 'inner' space)	7·60	8·83	12·0	10·4	8·84	
Q_{Na^+} (μl. slowly exchanging Na^+/mg. dry wt. of tissue/hr.)	±26·0	±28·3	±32·3	±49·1	±52·8	

* Sodium content of tissue before incubation = 63·5 μmoles/g. tissue.

cellular sodium and if its concentration was similar to that of the medium, then the space occupied by it would be 48·4 % of the tissue and will be called the 'outer' space of the tissue. This contrasts with the 'inulin' space of kidney-cortex slices of 26 % (Robinson, 1950). It is of interest that the size of the 'outer' space was 40 % of the tissue anaerobically at 37° and only 24·5 % at 0°. The remaining 'inner' space therefore contained the amounts of sodium shown in Table 2 and as expected there was least sodium in the 'inner' space of the tissue in aerobic conditions at 37° and most when the energy supply was reduced either by 2:4-dinitrophenol, anoxia or incubation at 0°.

In this paper 'turnover-rate' means the number of ions exchanging/min./100 ions present; 'exchange-rate' means the μmoles of ions exchanging/min./g. tissue. The turnover-rates of the sodium in the 'inner' spaces have been calculated using the formula (problem 1, case 3) of Stewart (1953). The rates were the same in all the aerobic conditions at 37°, despite large differences in the sodium content. The turnover-rate in anaerobic conditions was 25 % lower than that in aerobic conditions, whilst at 0° the rate was 45 % lower. The exchange-rates (in μmoles/min./g. tissue contained in the 'inner' space) at 37° were approximately proportional to the amount of sodium present. These results mean that at 37° the turnover-rate in the steady-state was largely independent of the amount of sodium in the tissue and hence of the energy supply (cf. Table 6).

Exchange of the potassium of kidney-cortex slices on incubation in vitro

Non-steady-state exchanges. Under aerobic and anaerobic conditions at 37° there was complete exchange between the potassium of the tissue and the medium (Table 3). The potassium was also completely exchangeable in the presence of 2 mM 2:4-dinitrophenol in the medium, or in the absence of substrate. As with sodium, the rate of exchange was more rapid aerobically than anaerobically. In contrast, the potassium content of the tissue was lower anaerobically than aerobically.

Steady-state exchanges at 0°. An unexpected result was obtained on aerobic and anaerobic incubation at 0° in medium containing $^{42}K^+$ from the start; about 20 and 40 %, respectively, of the tissue potassium had not exchanged even after 10 hr. The amount of exchange, however, increased with the period of incubation (Fig. 3) and was greater aerobically than anaerobically. These facts are in agreement with the assumption that there are two forms of intracellular potassium, one exchanging more rapidly than the other. The potassium content of the tissue did not alter much after the first 2 hr.

Table 3. *Changes in the distribution of* $^{42}K^+$ *between guinea pig kidney-cortex slices and medium during incubation at* 37°

(Slices of tissue (0·1-0·2 g.) incubated in 2·0 ml. of bicarbonate saline containing $^{42}K^+$ and 10 mM α-oxoglutarate; pH 7·4; gas phase 5 % CO_2 and either 95 % O_2 or 95 % N_2.)

Experimental conditions	Period of incubation (min.)	K^+ content of tissue after incubation* (μmoles/g. tissue)	Specific activity of K^+ in tissue (counts/min./μmole K^+). After incubation	Specific activity of K^+ in medium (counts/min./μmole K^+)		Ratio: (specific activity of K^+ in tissue/specific activity of K^+ in medium) × 100 (% exchange)
				Before incubation	After incubation	
Aerobic	2·0	42·2	722	2330	1680	43·0
	4·0	51·4	1040	2330	1900	54·8
	8·0	57·5	1250	2330	1750	71·4
	16	53·0	1470	2330	1745	84·4
	32	59·0	1630	2330	1800	90·6
	64	53·7	1510	2330	1610	94·0
	90	46·6	1640	2330	1630	100
	119	47·2	1550	2330	1540	100
	240	27·2	1580	2330	1540	103
	359	19·5	1780	2330	1760	101
Anaerobic	2·0	39·2	607	2800	2210	27·5
	4·0	32·7	926	2800	2210	41·9
	8·0	31·9	935	2800	1950	47·9
	16	29·2	995	2800	1900	52·4
	34	20·5	1280	2800	1950	65·7
	64	18·6	1380	2800	1970	70·1
	90	17·2	1350	2800	2000	67·5
	120	16·5	1440	2800	1950	74·0
	240	11·2	1690	2800	1640	103
	357	9·8	1860	2800	1880	99·0

* Potassium content of tissue before incubation = 76·2 μmoles/g. tissue.

of incubation and approximately steady-state conditions were established, with the tissue potassium content higher than that of the medium. The rates of exchange of the two forms of intracellular potassium can be represented by simple first-order kinetics. Aerobically the experimental points fit the curve

$$P = 37[1 - \exp(-0{\cdot}228t)] + 63[1 - \exp(-0{\cdot}00194t)];$$

anaerobically the points fit the curve

$$P = 37[1 - \exp(-0{\cdot}160t)] + 63[1 - \exp(0{\cdot}00069t)].$$

These curves were obtained from two different kinds of experiments; the fast rate from experiments in which the tissue was pre-incubated before the addition of radioactivity and the slow rate from experiments where the radioactivity was added at the beginning. These conditions were selected to make the overall time of incubation as short as possible. The slowly exchanging fractions of the tissue potassium had the very long times for half exchange of 6 hr. aerobically and 17 hr. anaerobically (Table 4). The rates for the more rapidly exchanging fractions of the tissue potassium at 0° are given in Table 4. In contrast to sodium, the rates for the rapidly exchanging fraction are far slower than would be expected for extracellular potassium.

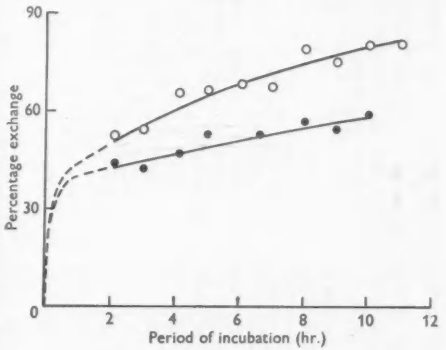

Fig. 3. The uptake of radioactive potassium by guinea pig kidney-cortex slices at 0°. About 0·15 g. tissue was incubated in 4·0 ml. of bicarbonate saline containing $^{42}K^+$ and 20 mM α-oxoglutarate at 0°; gas phase, 2·8% CO_2 and either 97·2% O_2 or 97·2% N_2; pH 7·4. ○, aerobic conditions; ●, anaerobic conditions. The curves represent the functions

$$P = 37[1 - \exp(-0{\cdot}228t)] + 63[1 - \exp(-0{\cdot}00194t)]$$

and

$$P = 37[1 - \exp(-0{\cdot}160t)] + 63[1 - \exp -0{\cdot}00069t)]$$

for aerobic and anaerobic conditions, respectively. P = the percentage exchange of the tissue potassium and t is the period of incubation in minutes. (For further details see text.)

Table 4. *Exchange of $^{42}K^+$ in guinea pig kidney-cortex slices during incubation at 0°*

(For measurements of the rates of the rapidly exchanging potassium, tissue (approx. 0·2 g.) was incubated in 2·0 ml. of bicarbonate saline containing 10 mM α-oxoglutarate, pH 7·4 (gas phase 2·8% CO_2 and either 97·2% O_2 or 97·2% N_2), for 30–40 min. and then placed for from 2 to 6 min. in a similar saline containing $^{42}K^+$. For measurements of the rates of the slowly exchanging potassium see Fig. 3. For further details see text.)

Experimental conditions	Period of incubation (min.)	Component of tissue K^+	Amount of K^+ in component (%)	K^+ content of tissue after incubation* (μmoles/g. tissue)	Rate constant of tissue K^+ ($min.^{-1}$)	Time for half-exchange of tissue K^+ (hr. min. sec.)	Turnover-rate of tissue K^+ (%/min.)	Exchange-rate of tissue K^+ (μmoles/min./g. tissue)	Q_{K^+} (μl. K^+ exchanging/mg. dry wt. of tissue/hr.)
Aerobic	40	Fast	37	30·9	0·228	0 3 02†	18·1	2·07	±15·7
Anaerobic				28·7	0·160	0 4 20†	12·7	1·35	±10·2
Aerobic	From 120 to 600	Slow	63	24·5	0·00194	5 57 0	0·155	0·0239	±0·212
Anaerobic				18·7	0·00069	16 40 0	0·0622	0·00732	±0·0656

* K^+ content of tissue before incubation = 76·1 μmoles/g. tissue.
† Calculated for K^+ in 200 mg. tissue.

If this fraction were contained in a space having the same potassium concentration as the medium, the space would be twice the volume of the whole tissue slices. Since this is impossible there must be at least two species of intracellular potassium at 0° in addition to extracellular potassium.

Steady-state exchanges at 37°. Preliminary experiments showed that in contrast to the results at 0°, all the potassium at 37° behaved as a single species. The turnover-rates were therefore measured according to Krebs *et al.* (1951). As at 0°, the extracellular potassium was neglected and this introduced an error of less than 6 %.

In spite of a twofold difference in the amount of potassium present, the turnover-rates under all conditions which were aerobic and at 37° were about the same (Table 5), whilst under anaerobic conditions the rate was 28 % lower. The similar turnover-rates found with and without 2:4-dinitrophenol are in contrast with the different amounts of potassium in the tissue. Thus, whilst the amount of ion is regulated by the energy supply, the turnover at 37° under aerobic conditions appears to be independent of it.

The 'outer' space of the slices contained sodium exchanging at a rate indistinguishable from that of the sodium in the extracellular space of the tissue. The potassium contents of the 'inner' spaces (Table 5) have been calculated on the assumption that this 'outer' space contains potassium also at the same concentration as the medium. These values show that there was a large reduction in the potassium content of the 'inner' space when the supply of energy from respiration was inhibited (with 2:4-dinitrophenol) or abolished (by anoxia).

Effect of oxygen uptake of kidney-cortex slices on the concentration gradients of sodium and potassium

Table 6 shows that at 37°, 10 mM α-oxoglutarate increased the Q_{O_2} of guinea pig kidney-cortex slices by 40 %. This concentration of α-oxoglutarate also led to a 15 % increase in the concentration gradients of sodium and potassium (Tables 2 and 5). The 60 % increase of the Q_{O_2} in the presence of 0·2 mM 2:4-dinitrophenol was accompanied by falls of 62 and 45 % in the concentration gradients of sodium and potassium (cf. Mudge, 1951*b*).

Relations between metabolism and sodium and potassium turnover

Table 7 gives the relations between the rate of respiration of kidney-cortex slices and the concomitant rates of exchange of sodium and potassium. The ratio, Q_{cation}/Q_{O_2}, was higher for respiration with endogenous substrate than with α-oxoglutarate, and much higher than the ratio in the presence of 0·2 mM 2:4-dinitrophenol. However, Tables 2 and 5 show that the turnover-rates of

Table 5. *Steady-state turnover-rates of* $^{42}K^+$ *in guinea pig kidney-cortex slices during incubation at* 37°

(Tissue (0·1–0·2 g.) incubated in 2·0 ml. of bicarbonate saline containing 10 mM α-oxoglutarate, pH 7·4 (gas phase, 5 % CO_2 and either 95 % O_2 or 95 % N_2), for 35–40 min. and transferred to a similar saline containing $^{42}K^+$ for from 2 to 6 min. Radioactivity of medium was measured before and after incubation. Turnover-rates calculated using formula of Krebs *et al.* (1951). For further details see text.)

Experimental conditions	K^+ content of tissue after incubation* (μmoles/g. tissue)	K^+ content of part of tissue not occupied by rapidly exchanging Na^+ (μmoles/g. tissue contained in 'inner' space)	Ratio: K^+ content in slowly exchanging Na^+ space/K^+ concentration in medium	Rate constant of tissue K^+ ($min.^{-1}$)	Time for half-exchange of tissue K^+† (min.)	Turnover-rate of tissue K^+ (%/min.)	Exchange-rate of tissue K^+ (μmoles/min./g. tissue)	Q_{K^+} (μl. K^+ exchanging/mg. dry wt. of tissue/hr.)
Aerobic	76·3	143	25·1	0·206	3·37	15·9	12·1	±80·0
Aerobic without added substrate	64·2	128	22·5	0·206	3·37	15·6	10·0	±66·2
Aerobic with 0·2 mM 2:4-dinitrophenol	34·6	78·4	13·8	0·206	3·37	16·9	5·85	±39·3
Anaerobic	23·1	34·7	6·1	0·145	4·78	11·5	2·66	±21·0

* Potassium content of tissue before incubation = 78·5 μmoles/g. tissue.
† Measured when the amount of K^+ in the medium was the same as the amount of K^+ in the tissue.

sodium and potassium remained nearly identical in these aerobic conditions. The lowered efficiency in the presence of α-oxoglutarate and 2:4-dinitrophenol is therefore apparently due to increments of respiration (Table 6) which do not affect the turnover-rates.

Table 6. *Oxygen uptake by guinea pig kidney-cortex slices*

(Slices of tissue (approx. 0·15 g.) placed in 4 ml. of phosphate saline (medium III of Krebs, 1950, without the addition of organic acids), gassed with O_2. CO_2 was absorbed by 0·2 ml. 2N-NaOH in the centre well and Q_{O_2} was measured with standard Warburg manometers.)

Additions to saline	Temp. (°)	Q_{O_2} (μl./mg. dry wt./hr.)
None	37	−16·8
10 mM α-oxoglutarate	37	−23·6
10 mM α-oxoglutarate and 0·2 mM 2:4-dinitrophenol	37	−37·8
10 mM α-oxoglutarate	0	−0·68
None	0	−0·54

Table 7. *Quotients relating the rate of respiration of guinea pig kidney-cortex slices to the rates of exchange of sodium and potassium*

(Calculated from results in Tables 2 and 4–6.)

Temp. (°)	Experimental conditions	Ratio: $\pm\frac{Q_{cation}}{Q_{O_2}}$ Na^+	Ratio: $\pm\frac{Q_{cation}}{Q_{O_2}}$ K^+
37	Aerobic, with 10 mM α-oxoglutarate	1·10	3·39
	Aerobic, no added substrate	1·69	3·47
	Aerobic, with 10 mM α-oxoglutarate and 0·2 mM 2:4-dinitrophenol	0·86	1·04
	Anaerobic	∞	∞
0	Aerobic	77·7	0·31* 23†
	Anaerobic	∞	∞

* This refers to the slowly exchanging part of the tissue K^+.

† This refers to the rapidly exchanging part of the tissue K^+.

The effects of oxygen and temperature on the turnover-rates of sodium and potassium are shown in Table 8. The turnover-rates in aerobic conditions were 30–40% higher than those in anaerobic conditions. The effect of temperature on the slowly exchanging part of the tissue potassium was very large, the increase aerobically being 104-fold and anaerobically 184-fold.

DISCUSSION

The exchangeability of the sodium and potassium of guinea pig kidney-cortex slices

Effects of respiration. It is useful to distinguish between the mechanisms which cause net movements of ions and those which maintain steady-state exchanges. These may be identical or different (Davies & Krebs, 1952).

Net movements of sodium into and of potassium out of the cell are in accordance with the concentration gradients and are unlikely to require energy supplies. However, the reverse process, the net movements of these cations in opposite directions against concentration gradients, must require energy whatever the electrical potential difference across the cell wall. Much work with isolated tissue slices has, in fact, shown that this process requires a supply of energy from respiration.

Respiration is also required to maintain a steady-state in the tissue in which the sodium and potassium concentrations are approximately physiological (Terner, Eggleston & Krebs, 1950; Krebs *et al.* 1951; Mudge, 1951*a*, *b*; Aebi, 1953; Whittam & Davies, 1953*b*). Changes in the metabolic state of the tissue lead to the establishment of steady-states with different sodium and potassium concentrations. Since in all these steady-states there is no net transport of ions but simply a to-and-fro exchange, it is possible that the steady-state turnover could take place without a direct energy supply. Such a turnover of ions occurs, e.g. between the sodium form of a cation-exchange resin in a neutral, dilute

Table 8. *Effect of oxygen and increase of temperature on the turnover-rates of sodium and potassium in guinea pig kidney-cortex slices*

(Calculated from results in Tables 2, 4 and 5.)

Cation	Ratio: turnover-rate in aerobic conditions/turnover-rate in anaerobic conditions: 0°	37°	Ratio: turnover-rate at 37°/turnover-rate at 0°: Aerobic	Anaerobic	Ratio: increase of turnover-rate due to O_2 at 37°/increase of turnover-rate due to O_2 at 0°
Na^+	1·00	1·34	1·81	1·35	>20‡
K^+	2·5* 1·42†	1·38	104·0* 0·88†	184·0* 0·91†	47* 0·82†

* This refers to the slowly exchanging part of the tissue K^+.

† This refers to the rapidly exchanging part of the tissue K^+.

‡ This was calculated after making allowance for the maximum possible error in the measurement of the turnover-rates.

solution of sodium salts, in Donnan systems and in 'exchange diffusion' as suggested by Ussing (1949). An alternative mechanism is that concentration gradients are maintained by 'active', i.e. energy-fed transport, which counteracts the effects of 'leakage' (cf. Krebs *et al.* 1951).

The results presented here of measurements of the uptake of radioactive sodium and potassium ions by isolated slices of guinea pig kidney-cortex in various steady-state conditions have shown that about 75 % of the aerobic turnover-rates of both these cations are apparently independent of respiration, although the maintenance of the physiological concentration gradients of these ions certainly depends on respiration.

The absence of effect of 2:4-dinitrophenol on the aerobic turnover-rates suggests that the effects of respiration are not mediated by high-energy phosphate bonds, whilst Table 8 shows that there is no direct proportionality between respiration and the turnover-rates.

Effects of temperature. At 37° all the potassium was uniformly exchangeable and the most remarkable effect of incubation at 0° was the appearance of a major fraction of intracellular potassium with a very slow turnover-rate. Similar results were found by Harris (1952) who showed that 20 % of the potassium of frog muscle exchanged very slowly at 0°, and by Mudge (1952) who found that 40 % of the potassium of rabbit kidney-cortex slices was not exchangeable even after 4 hr. incubation under anaerobic conditions at 25°. This effect of incubation at 0° on the potassium turnover of kidney-cortex slices cannot be directly related to respiration because it also occurred anaerobically.

At 0°, however, the sodium in the 'inner' space of the tissue exchanged far faster than the slowly exchanging fraction of the intracellular potassium. It is therefore unlikely that the slowness of this potassium turnover is due to a non-specific decrease in permeability.

Extracellular space of kidney-cortex slices

A large fraction of the tissue sodium exchanged at very rapid rates and was indistinguishable from the sodium in the spaces outside the cells. If this sodium is contained in a space with the same sodium concentration as the medium, it would occupy about 50 % of the tissue at 37°. This 'outer' space would be 24·5 % at 0° which compares well with the 'inulin' space of 26 % found by Robinson (1950).

The high values of the 'outer' space at 37° suggest that the barrier to the exchange of the rest of the sodium is not the outer cell membrane but may lie within the cell. In fact, this barrier may be associated in part with mitochondria which may be concerned in active transport (Bartley & Davies, 1952, 1954; Macfarlane & Spencer, 1953; Spector, 1953; Stanbury & Mudge, 1953). At 0°, with much-reduced metabolism, the rapidly exchanging fraction of the tissue sodium is the same as the 'inulin' space. It may be that the apparent extracellular space of other tissues is also affected by metabolism and the method of measurement.

SUMMARY

1. The uptake of radioactive sodium by aerobic guinea pig kidney-cortex slices in a steady-state at 37° can be represented by the empirical function

$$P = 76[1 - \exp(-2{\cdot}65t)] + 24[1 - \exp(-0{\cdot}151t)],$$

where P is the percentage exchange, and t is the time in minutes. Anaerobically at 37° the function is

$$P = 55[1 - \exp(-4{\cdot}45t)] + 45[1 - \exp(-0{\cdot}134t)],$$

and at 0°, aerobically and anaerobically,

$$P = 34[1 - \exp(-8{\cdot}28t)] + 66[1 - \exp(-0{\cdot}0954t)].$$

This means that sodium exchanged as at least two fractions and that both the relative amounts and the turnover-rates of the fast and slow fractions of tissue sodium changed with temperature and rate of respiration.

2. The rapidly exchanging tissue sodium apparently occupied an 'outer' space of about 50 % of the tissue volume at 37° and 25 % at 0°.

3. All the tissue intracellular potassium exchanged uniformly at 37° both aerobically and anaerobically with rate constants of 0·206 and 0·145 min.$^{-1}$, respectively. In contrast, the uptake of radioactive potassium at 0° in a steady-state can be represented by

$$P = 37[1 - \exp(-0{\cdot}228t)] + 63[1 - \exp(-0{\cdot}00194t)],$$

and anaerobically at 0° by the function,

$$P = 37[1 - \exp(-0{\cdot}160t)] + 63[1 - \exp(-0{\cdot}00069t)].$$

This means that at 0° the intracellular potassium exchanged as at least two fractions.

4. Respiration increased the turnover-rates (% per min.) of sodium and potassium at 37° by only 34 and 42 %. Thus, about 75 % of the aerobic turnover-rates are independent of respiration. The turnover-rates in O_2 were not affected by absence of substrate or the presence of 2:4-dinitrophenol despite major changes in the respiration rate and the amounts of cations present.

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Dietary Carotene and the Degree of Esterification of Vitamin A in the Milk and Blood of Cows

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In a previous paper (Chanda & Owen, 1952) the authors showed that depriving cows of carotene caused an increase of the vitamin-A alcohol in their milk and that this increase was enhanced by imposing thyroxine treatment simultaneously with the carotene deprivation. These increases were interpreted as indicating a draft by the cow from the stores of vitamin A in the liver. In the previous paper the effects of carotene deprivation on the composition of milk were studied but no analyses of blood were made. In the present experiment the β-carotene, vitamin A ester and vitamin-A alcohol were studied in both the blood and milk of a further four cows on diets which provided intakes of carotene varying from a mere trace to more than 2000 mg./cow/day.

EXPERIMENTAL

The experiment lasted 20·5 weeks and was divided into seven periods, all except the last of which are shown in Fig. 2. In periods 1, 3 and 6, each of four Ayrshire cows received diet 1, which was free from carotene and consisted of (parts by weight): oats, 6; bran, 1; field beans, 2; decorticated-earthnut meal, 1. Diet 2, which was fed during period 2, consisted of: oats, 18; beans, 12; dried grass, 9; lucerne meal, 4. In periods 4 and 5, diet 3 was fed. It consisted of: oats, 18; beans, 12; dried grass, 7; lucerne meal, 5. Diet 1 contained no carotene, whilst diets 2 and 3 contained carotene as a natural constituent of the dried grass and lucerne meals. The concentrate ration for each period was prepared in a single batch. On all diets, straw and beet pulp were fed as roughage. As in a previous experiment (Chanda, Clapham, McNaught & Owen, 1952), analyses showed that faecal carotene became negligible after 7 to 8 days of carotene deprivation.

In 24 hr. representative samples of Sunday's and Wednesday's milk, the partition of vitamin A between alcohol and ester and the content of carotene were determined. On the last day of each period and also in the middle of period 6 these same determinations were made on the blood of each

Table 1. *Carotene intake and carotene content of production rations*

Cow no.	Period	Food intake* (kg./day)	Carotene (mg./100 g. food)	Carotene intake (mg./day)
1	2	9·05	2·96	268
	4	10·50	5·26	552
	5	10·50	6·28	659
	7†	9·00	22·61	2035
2	2	9·07	2·96	269
	4	10·29	5·26	541
	5	10·50	6·28	659
	7†	9·50	22·61	2148
3	2	9·04	2·96	268
	4	10·50	5·26	552
	5	10·50	6·28	659
	7†	8·70	22·61	1967
4	2	8·13	2·96	241
	4	8·77	5·26	461
	5	8·47	6·28	532
	7†	8·30	22·61	1877

* Not including oat straw and beet pulp fed according to appetite.
† Grazing.

of the animals. During each of the carotene periods, 2, 4 and 5, samples of food were analysed every 4 days for carotene, and from the known weight of food eaten the carotene intake was calculated. At the conclusion of the experiment, in period 7, the four cows were used by the Animal Husbandry Department of this Institute for a grazing trial. From the intake of grass computed by them and from its carotene content the carotene intake during period 7 was calculated. It will be seen that the cows were allowed intakes of carotene which increased as the experiment progressed (Table 1). Carotene intakes varied from a mere trace in periods 1, 3 and 6 to about 2000 mg./day in period 7. The intake in periods 4 and 5 (about 550 mg./day) was twice as great as during period 2.

Methods of analysis

These were as described in earlier papers (Chanda, Owen & Cramond, 1951; Chanda & Owen, 1952). The procedure of Morton & Stubbs (1946) was used to correct both the milk and blood vitamin A for irrelevant absorption. Fig. 1 shows the extent of this irrelevant absorption for blood. The fat was extracted from the blood serum by the method of Yudkin (1941) but hexane was used as the solvent. The method of separation of vitamin-A ester in blood from vitamin-A alcohol and the method of separation of β-carotene from total carotenoids by chromatography on alumina were as described by Chanda & Owen (1952) for milk.

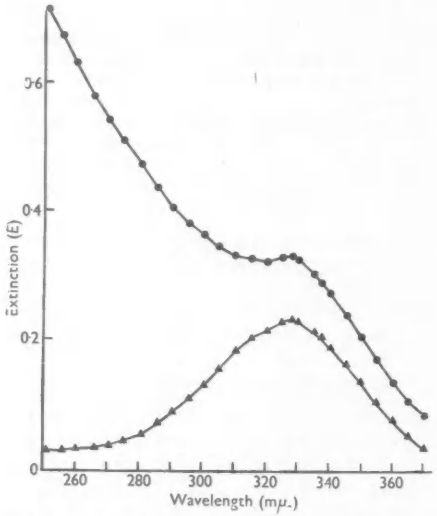

Fig. 1. The absorption spectrum of vitamin A in cow blood. Upper curve uncorrected, lower curve corrected for irrelevant absorption.

RESULTS

Composition of milk

The effects on the milk of the dietary treatments are shown in Tables 2 and 3 and in Fig. 2. As was expected, the amount of β-carotene in the milk

Table 2. *Average milk yield and average percentage of fat in the milk of each period, together with the concentration of β-carotene in the fat*

(β-Carotene is given as μg./g. fat.)

Period no.	1. Carotene-free			2. Carotene			3. Carotene-free			4. Carotene			5. Carotene			6. Carotene-free		
Cow no.	Milk yield (kg./day)	Fat (%)	Carotene	Milk yield (kg./day)	Fat (%)	Carotene	Milk yield (kg./day)	Fat (%)	Carotene	Milk yield (kg./day)	Fat (%)	Carotene	Milk yield (kg./day)	Fat (%)	Carotene	Milk yield (kg./day)	Fat (%)	Carotene
1	14·07	3·41	2·54	11·28	3·53	4·42	9·26	3·22	3·32	8·34	3·24	5·90	7·85	2·88	8·80	6·19	3·16	3·42
2	13·44	3·43	2·35	13·22	3·72	4·62	13·40	3·70	3·00	13·88	3·42	5·44	12·40	3·58	7·12	11·47	3·60	2·68
3	15·46	3·69	2·25	14·18	3·90	4·16	13·76	3·72	2·86	14·12	3·66	5·04	13·65	3·53	7·26	11·74	3·78	2·78
4	16·27	3·04	2·87	16·65	3·18	5·79	16·04	2·90	3·23	15·75	3·15	5·73	13·23	3·63	8·07	12·12	3·19	3·08

Table 3. *Minimum vitamin-A ester and corresponding β-carotene and vitamin-A alcohol during deprival of β-carotene (periods 1, 3 and 6) and maximum vitamin-A ester and corresponding β-carotene and vitamin-A alcohol during re-alimentation with β-carotene*

(β-Carotene is given as μg./100 ml. milk and vitamin A as i.u./100 ml. milk.)

Cow no.	Period 1			Period 2 (carotene)			Period 3			Period 4 (carotene)			Period 5 (carotene)			Period 6		
	β-Carotene	Vitamin A		β-Carotene	Vitamin A		β-Carotene	Vitamin A		β-Carotene	Vitamin A		β-Carotene	Vitamin A		β-Carotene	Vitamin A	
		Ester	Alcohol		Ester	Alcohol		Ester	Alcohol		Ester	Alcohol		Ester	Alcohol		Ester	Alcohol
1	6	54	12	16	86	6	7	46	12	25	97	5	25	97	7	6	40	13
2	6	50	13	17	80	7	6	43	12	25	106	5	26	104	5	5	42	13
3	6	50	13	19	80	8	5	56	15	24	99	7	25	100	6	5	41	15
4	6	47	15	20	75	7	5	40	15	25	97	8	26	103	6	4	34	15

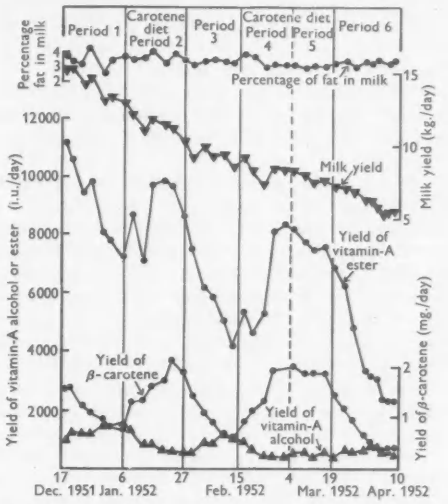

Fig. 2. Yields of milk, of carotene, vitamin-A ester and vitamin-A alcohol in milk, and percentage of fat in milk of cow no. 1.

reflected the β-carotene intake. On the carotene-free diet the β-carotene in the milk decreased gradually in period 1 and more rapidly in periods 3 and 6. In periods 2 and 4, re-alimentation with carotene caused rapid increases of β-carotene in the milk. Fig. 2 shows the milk yield, the percentage of fat in the milk and the outputs in the milk of vitamin-A ester, vitamin-A alcohol and β-carotene for cow no. 1, which in this and all other respects was typical. For brevity, therefore, the graphs for the other three cows have been omitted. Data in Tables 1–5 show that the behaviour of all four cows was uniform. The general decline (Fig. 2) of the outputs of vitamin-A ester and β-carotene is a reflexion of the natural decline of the milk yield. Throughout the experiments and for all the milk samples examined from all four cows, the percentage of β-carotene in the total milk carotenoids ranged from 76 to 92 averaging 82·4 ± 1·9 S.E. The non-carotene carotenoids were 13, 28, 14, 10, 10 and 11 μg./100 ml. milk for periods 1–6, respectively, each figure being the average of four concordant averages for each of the four cows. In Table 2, the average milk yield, the average percentage of fat in the milk and the average concentration of β-carotene in the milk fat are recorded for the four cows. In Table 3, the minima of vitamin-A ester in the milk during deprival of carotene in periods 1, 3 and 6 and maxima of vitamin-A ester during re-alimentation with β-carotene are recorded, together with the values of β-carotene, vitamin-A alcohol and

fat in the samples of milk which showed these maximum and minimum values. It was noticed that, when vitamin-A ester was maximal, β-carotene was also maximal, and that minima of these two substances in the milk also coincided. In contrast, minima of vitamin A-alcohol in the milk coincided with maxima of vitamin-A ester and vice versa (Fig. 2).

Vitamin-A ester in milk. The amount of vitamin-A ester in the milk changed with diet in much the same way as did β-carotene (Fig. 2 and Table 3), decreasing markedly in periods 1, 3 and 6 when carotene was withheld from the diet, and increasing markedly on re-alimentation with carotene in periods 2 and 4. These changes were of similar magnitude in all four cows and were obvious, whether the results were expressed as yields per cow or as percentages of the milk or as percentages of the milk fat. Indeed this last statement was true of the changes of β-carotene and of vitamin-A alcohol also.

Relationships between vitamin-A ester and β-carotene and between vitamin-A ester and vitamin-A alcohol in milk. Comparison of the figures for successive periods in Table 3 and a reference at the same time to the trends in Fig. 2 show the dependence of both β-carotene and vitamin-A ester in the milk upon dietary β-carotene. Fig. 2 also shows that trends of vitamin-A alcohol were the reverse of those of β-carotene and vitamin-A ester. In confirmation of a previous finding of Chanda & Owen (1952), Fig. 2 shows that vitamin-A alcohol increased as β-carotene intake diminished, and decreased as β-carotene intake increased, thus producing the relationships between their maxima and minima already mentioned and illustrated in Fig. 2 and Table 3. The changes of vitamin-A ester in the milk were in the same direction as those of β-carotene, but the changes of vitamin-A alcohol were in the opposite direction from those of β-carotene. Thus, correlations between vitamin-A ester and β-carotene in the milk were large and positive, whilst, in contrast, correlations between vitamin-A ester and vitamin-A alcohol were almost equally large and were negative. The coefficients of these correlations, recorded in Table 4, are statistically very significant.

Table 4. *Correlations between vitamin-A ester and β-carotene and between vitamin-A ester and vitamin-A alcohol*

(Values are determined as i.u. (vitamin-A ester or alcohol) or μg. (β-carotene)/100 ml. whole milk.)

Cow no.	No. of paired observations	Coefficients of correlation: Vitamin-A ester × β-carotene	Coefficients of correlation: Vitamin-A ester × vitamin-A alcohol
1	35	+0·930	−0·862
2	35	+0·902	−0·829
3	35	+0·891	−0·783
4	33	+0·926	−0·821

Composition of blood serum

β-Carotene in blood serum. Table 5 shows how sensitive the blood serum was to changes of intake of β-carotene. For all four cows, blood carotene increased approximately in proportion to intake, and all of them showed the lowest carotene figures when they were eating the carotene-free diet (in periods 1, 3 and 6) so that in all the cows β-carotene in the blood was a fair indication of the carotene intake. Nevertheless, it is noteworthy that even after 3 weeks of depletion there was still an easily measurable amount of β-carotene in the blood.

Non-carotene carotenoids were present in the blood as some 10–20% of the total carotenoids. They were present in the blood serum in smallest concentration in periods 1, 3 and 6. In periods 2, 4, 5 and 7 their concentrations, like those of β-carotene, reflected the intake of greenstuff. In periods 1, 3 and 6 the blood serum contained 8, 12 and 10 units, respectively, of non-carotene carotenoids. In periods 2, 4, 5 and 7 it contained 23, 44, 53 and 100 units, respectively, each figure being the average of four concordant figures given by the four cows in each period. The figures were calculated on the same basis as was used for true β-carotene. These figures show that the passage of non-carotene carotenoids into the blood did not increase as rapidly with increasing intake of greenstuff as did the passage of true β-carotene into the blood (Table 5). Differences between periods in the non-carotene carotenoids in the blood were greater than the corresponding differences in the milk. This disparity between the blood and the milk probably indicates limitations of the permeability of both the intestinal and mammary epithelia to non-carotene carotenoids. Comparison of milk and blood β-carotene values in Tables 3 and 5 shows that similar considerations apply to β-carotene also.

Vitamin A in blood serum. Table 5 shows the concentrations of vitamin-A ester, of vitamin-A alcohol and of β-carotene in the blood of four cows in each of the experimental periods. It shows clearly the dependence of vitamin-A ester in the blood on the carotene intake. When the intake of β-carotene was zero, the blood vitamin-A ester was zero also. As the carotene intake increased so did the blood vitamin-A ester. The table shows that in respect of vitamin-A ester all four cows behaved similarly. As already mentioned, the effect of β-carotene intake on blood β-carotene was equally marked but, in contrast to vitamin-A ester, blood β-carotene did not become zero on zero intake. Blood vitamin-A alcohol behaved quite differently from either vitamin-A ester or β-carotene. The small changes which it showed bore no relation at all to the intake of β-carotene. Thus, although subjection of the cows to periods of deprival of dietary carotene had

Table 5. *Carotene deprivation and the concentrations of β-carotene, vitamin-A ester and vitamin-A alcohol in blood serum of the lactating cow*

Period no.	Duration (days)	Carotene* intake (mg./day)	β-Carotene (μg./100 ml. serum)				Vitamin-A ester (i.u./100 ml. serum)				Vitamin-A alcohol (i.u./100 ml. serum)			
			Cow no. 1	Cow no. 2	Cow no. 3	Cow no. 4	Cow no. 1	Cow no. 2	Cow no. 3	Cow no. 4	Cow no. 1	Cow no. 2	Cow no. 3	Cow no. 4
1	21	Trace	48	45	64	38	0	0	0	0	87·8	96·1	103·7	84·0
2	20	262	136	131	151	168	11·4	9·7	12·5	10·5	86·6	99·6	107·9	86·6
3	18	Trace	70	57	80	89	0	0	0	0	82·1	93·1	98·4	87·4
4	17	527	411	339	446	424	21·2	25·1	26·5	27·1	95·8	111·7	114·0	105·6
5	15	627	555	443	614	552	27·8	25·5	29·6	27·8	111·7	104·9	120·1	100·3
6*a*	13	Trace	93	92	119	101	0	0	0	0	107·9	115·5	117·0	113·2
6*b*	10	Trace	38	40	47	47	0	0	0	0	100·3	96·5	113·2	96·5
7	29	2007	867	822	944	1117	31·6	36·8	31·0	34·4	99·6	107·9	127·7	111·7

* Average for all four cows.

no effect on vitamin-A alcohol in the blood serum, it nevertheless caused a marked diminution of circulating β-carotene and caused vitamin-A ester to disappear altogether from the blood serum. At the conclusion of period 7, during which access to pasture enhanced the intakes of carotene from an average of 635 mg./cow/day in period 5 to an average of more than 2000 mg., the largest concentrations of vitamin-A ester and β-carotene in the blood occurred. These large changes of carotene intake had, however, no demonstrable effect on the vitamin-A alcohol in the blood serum (Table 5). Inspection of Table 5 shows that blood β-carotene increased much more with increasing intake of greenstuff than did vitamin-A ester. This was doubtless due to the efficient removal of vitamin A from the blood by the mammary gland or the liver or both.

DISCUSSION

These experiments confirm the earlier observation of Chanda & Owen (1952) that the metabolism of vitamin-A ester is distinct from that of vitamin-A alcohol and that as they exist in the blood serum these two substances are not in equilibrium with one another. Thus, Chanda & Owen (1952) found that deprivation of carotene caused a marked increase of both the concentration and the yield of vitamin-A alcohol in the milk. Such deprivation also caused simultaneous reductions of the concentrations of carotene and vitamin-A ester in the milk. These observations are confirmed by the present experiments. In the blood, the vitamin-A alcohol was unaffected by the large changes of intake of carotene. Since most of the vitamin A in the blood is in the form of the alcohol, it is not surprising that attempts to change the total vitamin A in the blood by means of dietary carotene are not successful (Klosterman, Bolin & Ford, 1951; Thomas & Moore, 1952; others quoted in a review by Owen, 1954). Chronic deprivation of carotene decreases the concentration of vitamin A in the blood (Eaton, Helmbolt, Jungherr, Carpenter & Moore, 1951; Eveleth, Bolin & Goldsby, 1949), but larger intakes of carotene do not necessarily increase it (Klosterman *et al.* 1951; Thomas & Moore, 1952). Apparently the stores of vitamin A in the liver need to be severely depleted for the cow to lose its ability to maintain the normal concentration of vitamin-A alcohol in the blood. By analogy with work from other laboratories on the metabolism of β-carotene and vitamin A in the rat, it is extremely probable that the action of thyroxine in the experiments of Chanda & Owen (1952) was to mobilize the reserves of vitamin A in the liver and to release them into the blood as vitamin-A alcohol. It is necessary to suppose that the power of the mammary gland to re-esterify vitamin A is limited. Without such

a hypothesis it is difficult to account for the increase of the free form of the vitamin in the milk, when, owing to deprivation of carotene, the cow is forced to use its liver reserves. When thyroxine causes these liver reserves to be mobilized at an enhanced rate, the limited power of the mammary gland to esterify vitamin A is placed even more in evidence (Chanda & Owen, 1952). Nevertheless, it must be supposed that the gland does esterify the vitamin, since the present experiments show that the ester form is absent from the blood of cows which are deprived of carotene, while the bulk of the vitamin A in the milk is still in the ester form (Table 5).

The present experiments confirm the observation of Chanda (1953) that vitamin-A ester occurs in cattle blood, and show that it reflects the intake of β-carotene, as indeed the experiments of Thompson, Ganguly & Kon (1949) would lead one to expect. In the absence of evidence as to the relative rates at which the mammary epithelium can take up vitamin-A alcohol and vitamin-A ester, it is not possible to say in which form the major part of the vitamin A originating from dietary carotene is absorbed by the mammary gland. β-Carotene is absorbed from the gut into the intestinal lymphatics (Thompson *et al.* 1949; Glover, Goodwin & Morton, 1948; Eden & Sellers, 1950) chiefly as vitamin-A ester. From these lymphatics the vitamin-A ester will pass to the venous system, to the lungs and then to the general body circulation, so that the liver will have no better chance of intercepting it than will the mammary gland.

SUMMARY

1. Lactating cows were fed, in alternate periods, carotene-free diets and diets containing carotene; the intakes of carotene in the successive carotene periods increased as the experiment progressed.

2. In confirmation of the authors' previous results, carotene deprivation caused the amount of vitamin-A alcohol in the milk to increase.

3. The concentration of vitamin-A alcohol in the blood serum was not affected by the removal of carotene from the diet.

4. Carotene deprivation caused vitamin-A ester to disappear completely from the blood serum. The ester reappeared when carotene was again fed.

5. It is concluded from these results and from earlier results with thyroxine-treated animals that increases in vitamin-A alcohol in the milk of cows indicate a transfer of reserves of vitamin A from the cow's liver to the milk.

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The Excretion of Kynurenine and 3-Hydroxykynurenine by Man

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3-Hydroxykynurenine was first shown to be a tryptophan metabolite in animals in experiments with pyridoxine-deficient rats (Dalgliesh, Knox & Neuberger, 1951; Dalgliesh, 1952*b*). Later, Makino, Satoh, Fujiki & Kawaguchi (1952) claimed that 3-hydroxykynurenine is responsible for the diazo reaction and Weiss urochromogen reaction shown by the urine of patients suffering from acute tuberculosis. This suggested that in acute tuberculosis the function of pyridoxine-deficient enzymes might be deranged, and the purpose of this work was to investigate this possibility. It was found that the excretion of hydroxykynurenine by man is unrelated to tuberculosis and occurs in many cases

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of fever of various origins. The excretory pattern of tryptophan metabolites found in fever is compared with that found when tryptophan is ingested by the normal human, and the significance of the results is discussed.

METHODS

Preparation of the urines. Overnight urine samples of approximately 200 ml. were obtained from patients having fever (taken as a body temperature exceeding 100° F). A portion was tested by the diazo reaction and the remainder, when appropriate, prepared for chromatography using methods described by Dalgliesh (1952*b*) and Charconnet-Harding, Dalgliesh & Neuberger (1953). Briefly, the urine was acidified with acetic acid, shaken with charcoal which had been deactivated with 4% stearic acid, the charcoal filtered off, washed with water, and the aromatic substances were eluted from the charcoal by 5 or 10% (w/v) aqueous phenol. Extracts from human, unlike rabbit and rat, urines deposited crystals if allowed to stand. These were isolated in some cases and identified as uric acid. Normally when precipitation occurred the precipitate was isolated and discarded. The phenol eluates, after filtration where necessary, were evaporated to dryness under N_2 at a low pressure and at about 50–60°. The residues were taken up in water for paper chromatography, any uric acid present remaining undissolved.

Paper chromatography (cf. Dalgliesh, 1952*b*). Samples of the urine concentrates were run overnight on paper chromatograms (descending technique) using the organic layer from a freshly prepared butanol:acetic acid:water mixture (4:1:5, v/v). Hydroxykynurenine was detected in particular by its characteristic yellow-green fluorescence under ultraviolet illumination, by the almost immediate formation of a brown-black spot on treatment with ammoniacal silver nitrate and by the pink colour given with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in dilute HCl). Kynurenine was detected in particular by its fluorescence and behaviour with Ehrlich's reagent. The R_F values of kynurenine and hydroxykynurenine were shown to agree with those of authentic specimens. The relative amounts were judged visually from the size and intensity of the spots. When appreciable amounts of hydroxykynurenine were present it could be clearly seen as a yellow spot on the untreated chromatogram. The method of use of the various reagents has already been described (Dalgliesh, 1952*b*). In addition, hydroxykynurenine reacted extremely rapidly with the ferric chloride ferricyanide reagent for phenols devised by Barton, Evans & Gardner (1952). Attempts to develop a reagent using tetrazolium salts were unsuccessful.

In view of the widespread use of Dent's (1948) 'map of the spots', the positions of kynurenine and hydroxykynurenine were determined on two-dimensional chromatograms run by the ascending technique in phenol and then collidine, using metal frames (Datta, Dent & Harris, 1950). Kynurenine and hydroxykynurenine corresponded closely in positions with phenylalanine and tryptophan, respectively. The two former could be distinguished by their fluorescence, but Ehrlich's reagent was inapplicable, owing to excessive background, unless collidine was removed by successive washing of the paper with ethanol and ether.

Weiss reaction and diazo reaction. The Weiss urochromogen reaction (production of a golden-yellow colour on treatment of the urine with dilute permanganate) was carried out according to the Merck Index (1940*a*). It is an unspecific reaction which might be given by many reducing substances, and is therefore not easy to interpret. For routine examination we used the diazo reaction, which calls for comment. The procedure used, based on procedures described in the Merck Index (1940*b*) was as follows: equal volumes (about 5 ml.) of urine and 0·5% (w/v) sulphanilic acid in approx. 0·5N-HCl were mixed, and 3–5 drops of a 0·5% (w/v) $NaNO_2$ solution added, followed by 6–8 drops of NH_3 solution (sp.gr. 0·880). The tube was then vigorously shaken. A positive reaction was one in which, after shaking, a pink or red colour appeared in the foam. Many substances of possible occurrence in urine, such as phenols, glyoxaline derivatives and pyrroles (cf. van den Bergh reaction) give in solution a red or orange colour with the diazo reagent, and in fact some degree of red coloration in the solution was found with all urines examined. Most of these on shaking gave a yellow foam quite distinct from the pink foam found when hydroxykynurenine was present. Some experiments were carried out with authentic hydroxykynurenine and with 3-hydroxyanthranilic acid. The latter contains the same aromatic system as hydroxykynurenine, and has been isolated from the urine of a tuberculous patient (Musajo, Spada & Coppini, 1952). It was thought likely that it would behave similarly to hydroxykynurenine in the diazo reaction. On submitting either hydroxykynurenine or hydroxyanthranilic acid to the diazo reaction, a red colour was produced in solution, but no foam was formed on shaking. On adding protein (a drop of serum) or detergent and again shaking, the hydroxykynurenine gave the typical pink foam, whereas the foam from the hydroxyanthranilic acid was yellow. The 'foam' method of carrying out the diazo reaction seems satisfactory for human urines. That this is fortuitous, however, is shown by the fact that almost any rabbit urine will give a positive result by this technique, even though no hydroxykynurenine can be detected chromatographically.

Feeding experiments. Two normal males took by mouth 10 g. DL-tryptophan as a slurry in water. Further water (about 1 l. altogether) was taken and urines were collected for the periods 0–8 and 8–24 hr. The urines were worked up and examined chromatographically as described above. In a further experiment a normal male was injected intravenously with 0·5 ml. typhoid vaccine. After a short while the body temperature started to rise, and 10 g. DL-tryptophan was taken by mouth as before. The body temperature remained at 101·4° F for about 12 hr., and urine excreted during this period was collected and examined.

RESULTS

The conclusion of Makino *et al.* (1952) that hydroxykynurenine is responsible for the diazo reaction is confirmed. Wherever a positive reaction was encountered the urine was found by paper chromatography to contain hydroxykynurenine, and no hydroxykynurenine was found in those urines showing a negative reaction. Altogether some hundred urines were examined, including normal controls. The results are summarized in Tables 1–3. Table 1 summarizes cases in which hydroxykynurenine excretion was observed, and shows that the excretion is not dependent on sex or drug

treatment. Table 2 summarizes all fever patients whose urine was examined chromatographically, whilst Table 3 similarly summarizes non-febrile cases. The only apparent common factor in the positive cases is the existence of fever. In Table 3 it will be observed that hydroxykynurenine excretion was not observed in acute thyrotoxicosis, in which the metabolic rate is raised without accompanying fever, or in various other non-febrile states associated with weight loss. Kotake & Tani (1952) have recently reported the excretion of hydroxykynurenine in insulin-treated diabetes. We have examined a large number of cases of insulin-treated diabetes (Table 3), and in no case was hydroxykynurenine excretion observed. In view of the marked tendency for diabetes to co-exist with other disorders it seems probable to us that the excretion observed by Kotake & Tani may be ascribed to fever due to complications, and not to diabetes as such.

When DL-tryptophan was taken by mouth by the normal human, considerable amounts of kynurenine were excreted in the urine, but no hydroxykynurenine. It is of interest that D-kynurenine was excreted as well as the L-isomer (the two are distinguishable chromatographically; Dalgliesh, 1952*a*, *b*), though the L-isomer predominated. Similar conversion of D-tryptophan into D-kynurenine has been observed *in vivo* in the rabbit (Kotake & Ito, 1937), rat (Dalgliesh, 1952*b*) and man (Langner & Berg, 1953), although the conversion does not take place with isolated systems (Knox & Mehler, 1950). Kynurenine excretion was also observed in some of the febrile cases excreting hydroxykynurenine. With patients excreting large amounts of hydroxykynurenine, a much smaller amount of kynurenine excretion occurred, whereas when hydroxykynurenine excretion was low, no kynurenine could be detected.

Ehrlich's *p*-dimethylaminobenzaldehyde reagent revealed many indolic compounds which were not investigated in detail. In all cases some tryptophan was excreted. Tryptophan is known to be normally excreted by the human (e.g. Albanese & Frankston, 1945). The four principal Ehrlich-reacting compounds otherwise encountered are briefly summarized in Table 4 (*A–D*). In almost all urines, two fast-running indolic compounds, *A* and *B*, appeared. One or both of these may correspond to the ether-soluble tryptophan metabolite of Sarett & Goldsmith (1949) or to the 'aberrant metabolite' of Albanese (1944) and Albanese & Frankston (1944).

Table 1. *Excretion of hydroxykynurenine in fever, showing independence of sex and drug treatment*

Sex	Diagnosis	Drug treatment
M.	Tuberculosis	Streptomycin and *p*-aminosalicylic acid
M.	Tuberculosis	Streptomycin and *p*-aminosalicylic acid
F.	Tuberculosis	*iso*Nicotinic acid hydrazide and *p*-aminosalicylic acid
M.	Tuberculosis	*iso*Nicotinic acid hydrazide and streptomycin
F.	Bronchitis	Penicillin and ephedrine
M.	Bronchitis	Penicillin and aminophylline
M.	Syphilis and bronchitis	Penicillin
F.	Pneumonia	Penicillin and codeine
F.	Influenza	None
F.	Lupus erythematosus	Cortisone
M.	Cor pulmonale and pleurisy	Procaine penicillin
M.	Epilepsy and paroxia	None
M.	Leukaemia	None

Table 2. *Summary of fever cases in which the urine was examined chromatographically*

Diagnosis	No. of cases: Excreting hydroxykynurenine	No. of cases: Not excreting hydroxykynurenine
Tuberculosis (excluding mild cases)	4	2
Bronchitis	2	2
Syphilis and bronchitis	1	—
Influenza and bronchitis	—	1
Pneumonia	1	2
Pneumonia and pernicious anaemia	—	1
Pleural effusion	—	1
Influenza	1	—
Bronchopneumonia	—	1
Lupus erythematosus	1	1
Cor pulmonale	1	1
Epilepsy and paroxia	1	—
Jaundice	—	1
Leukaemia	1	—
Tonsilitis	—	1
Thrombophlebitis	—	1
Peptic ulcer and pyrexia due to malignancy	—	1
Coronary thrombosis	—	2

Table 3. *Non-febrile cases in which no hydroxykynurenine excretion was observed*

Diagnosis	No. of cases
Acute thyrotoxicosis	6
Sarcoidosis	1
Prolapsus intervertebralis	2
Myasthenia gravis	1
Polyneuritis	1
Psychotic depression	1
Muscular dystrophy	1
Rheumatoid arthritis	1
Bronchogenic carcinoma	1
Coronary infarct	2
Diabetes and muscular atrophy	1
Diabetes	43

Table 4. *Behaviour of some unknown tryptophan metabolites, and of some known compounds*

(R_F values from descending chromatograms on Whatman no. 1 paper. Solvent mixture; butanol:acetic acid:water; 4:1:5, v/v.)

Substance	Colour with Ehrlich's reagent	Mean R_F value
A	Blue	0·88
B	Blue	0·75
C (? indican)	Orange-red, developing slowly	0·44
D	Blue	0·33
Indoleacetic acid	Blue	0·88
N^a-Acetyltryptophan	Blue	0·86

The slower-running of the pair, substance *B*, is present in the greater amount. Substance *A* showed some resemblance in chromatographic behaviour to both indoleacetic acid and N^a-acetyltryptophan (Table 4), though slight differences appeared on paper electrophoresis. As indoleacetic acid is known to occur in human urine (e.g. Pacheco, 1953), *A* might represent this substance. Substance *D*, which only appeared occasionally, runs more slowly than tryptophan and probably contains a fairly hydrophilic system. Substance *C* appeared often, but not always, and from its chromatographic behaviour we consider that it almost certainly represents indican. It slowly gives an orange-red or brown colour with Ehrlich's reagent; indican is well-known to give such a colour with *p*-dimethylaminobenzaldehyde. If the chromatogram is sprayed with dilute acid and heated, substance *C* gives a blue or green-blue spot, whereas similar treatment with alkali has no effect. Indican is readily hydrolysed by acid (the resultant indoxyl being oxidized to the blue indigo) but is stable to alkalis. Substance *C* on chromatograms gives a yellow-brown colour on treatment with nitrous fumes, couples slowly with diazotized sulphanilic acid to give a red colour, but does not give a ninhydrin reaction nor reduce ammoniacal silver nitrate. All these are the expected properties for indican, but unfortunately we had no authentic material for direct comparison.

DISCUSSION

Hydroxykynurenine is not excreted in all cases of fever. It was observed in almost half of the cases examined, but the patients available to us were those in a general hospital. They did not include any with infectious diseases (e.g. measles, typhoid fever, scarlet fever, etc.), which have long been recognized to give rise to diazo-positive urines. Moreover, with drug treatments now available, patients are not often febrile for long after admission to hospital, and the fever may in some cases have wholly or partly dropped before collection of urines for examination. Thus it is likely that if all types of fever patients were examined during the course of an untreated fever, an appreciably higher proportion would be found to be excreting hydroxykynurenine.

The amount of hydroxykynurenine excreted in some cases seemed much too large to have been derived from dietary tryptophan, and we consider it likely that the excretion is due to a high rate of breakdown of body-proteins 'flooding' the enzyme systems responsible for tryptophan breakdown. Albanese & Frankston (1945) presented evidence that the small amount of tryptophan normally found in human urine is derived from protein breakdown and not from the diet. We have noticed in the present work that hydroxykynurenine excretion is accompanied by a considerably increased excretion of uric acid as is to be expected during general tissue breakdown. Increased uric acid excretion was also observed in thyrotoxicosis, though here it was not accompanied by hydroxykynurenine excretion. Owing to individual variations in the rate of protein breakdown and in the size and activity of the available enzyme pool, it is to be expected that the threshold for excretion of a substance such as hydroxykynurenine would not be exceeded in all cases.

The fact that hydroxykynurenine, but little or no kynurenine, is excreted in fever, whilst the normal human taking an excess of tryptophan by mouth excretes kynurenine and not hydroxykynurenine, is very striking. The place of kynurenine and hydroxykynurenine in tryptophan metabolism is summarized in Fig. 1 (cf. Dalgliesh, 1951, 1952*b*). (It is possible that kynurenine and hydroxykynurenine themselves are not the normal intermediates but that derivatives such as phosphates (cf. Charconnet-Harding *et al.* 1953) are involved. These, if formed, might well be excreted as kynurenine or hydroxykynurenine. In this discussion the terms kynurenine and hydroxykynurenine are considered to cover possible intermediates at the kynurenine and hydroxykynurenine levels of oxidation, respectively.)

Both kynurenine and hydroxykynurenine are split by the same enzyme, kynureninase. It has been shown by Knox (1953) that in the rat the potential rate of formation of kynurenine exceeds the potential rate of breakdown by kynureninase. The first step in tryptophan oxidation is moreover adaptive, increasing this difference between the potential rates of kynurenine formation and breakdown. A similar state of affairs may well exist in man. Besides being attacked by kynureninase, any kynurenine formed can be removed in three other ways: (*a*) by oxidative deamination or transamination to give, after ring closure, kynurenic acid, (*b*) by conjugation involving one or more of the so-called 'detoxication' mechanisms, and (*c*) by ring

oxidation to give hydroxykynurenine. The absence of significant amounts of kynurenic acid in human urine suggests that reaction (*a*) is quantitatively of slight importance. The absence of appreciable amounts of conjugated derivatives of kynurenine (or hydroxykynurenine) from our chromatograms suggests that (*b*) is also of slight importance. The absence of any appreciable amount of anthranilic acid or derivatives from human urine suggests that kynurenine is not normally present in sufficient amount (or possibly not at the correct site) for the action of kynureninase to be quantitatively significant. The greater part of normal kynurenine would therefore appear to go by route (*c*) to hydroxykynurenine. The results obtained on feeding tryptophan to the normal man are consistent with this view. The large excretion of kynurenine would indicate its formation at such a rate that the enzymes normally further metabolizing it are flooded, so that kynurenine is excreted as such. The absence of hydroxykynurenine excretion would indicate that hydroxykynurenine is further metabolized as fast as it is formed, which would also suggest that the enzyme converting kynurenine into hydroxykynurenine is not adaptive.

More difficult to explain is the fact that when hydroxykynurenine is excreted in fever the amount is always greatly in excess of any kynurenine that might be present. This indicates that if the apparent block in fever is simply due to one enzyme being present in limiting amount, this block occurs after the hydroxykynurenine stage. It is of interest that not more than possible traces of conjugated derivatives (sulphate, etc.; cf. Dalgliesh, 1952*b*) of hydroxykynurenine were observed. This suggests that either the human cannot deal with an excess of hydroxykynurenine by using 'detoxicating' mechanisms available to the rat, or that the hydroxykynurenine is excreted before it can be attacked by the appropriate enzymes. As sulphate and glucuronide excretion normally occur in man, the second explanation seems the more likely.

In a preliminary communication (Dalgliesh & Tekman, 1953) we advanced as possible explanations (*a*) that the enzymes involved were markedly temperature-dependent, so that, for example, the relative rates of two successive reactions might have been altered by change of temperature, and (*b*) that pathways for metabolism of endogenous and exogenous tryptophan differed. We also suggested that an alternative pathway from tryptophan to hydroxykynurenine might have been involved, possibly via 7-hydroxytryptophan. As 7-hydroxytryptophan has now been synthesized (Ek & Witkop, 1953) and shown not to be attacked by the enzyme system converting tryptophan into kynurenine (Udenfriend, Clark & Titus, 1953) such an alternative pathway seems unlikely. It is more probable that any difference in the metabolism of endogenous and exogenous tryptophan is due to the liberation of intermediates in amounts, and/or at sites, of varying favourability for further metabolism. In order to investigate the possible temperature-dependence (or the possible adaptive nature) of steps in tryptophan degradation, it would be necessary to do enzymic experiments. No record has yet appeared of a successful attempt to isolate the enzyme system bringing about the hydroxylation of the aromatic ring of kynurenine.

The explanation of the results obtained in the present work is therefore still in doubt. When tryptophan was given by mouth to a normal human in whom an artificial fever had been engendered, no hydroxykynurenine was excreted, suggesting that our results are due to a difference between exogenous and endogenous tryptophan metabolism. On the other hand, the fever was of short duration, and in a few patients with sustained fever who were followed for some days, it was found that there was sometimes a delay after onset of fever before the appearance of hydroxykynurenine in the urine. Hydroxykynurenine excretion always ceased with loss of fever. Although our results are inconclusive, they are reported at this stage as it is felt that further investigation must await a method for the quantitative determination of hydroxykynurenine. Unfortunately no method at present exists for the specific estimation of this substance in the type of complex mixture obtaining in urine.

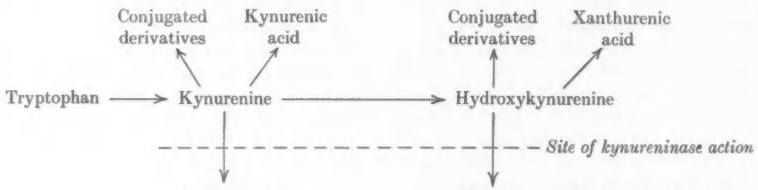

Fig. 1. Summary of tryptophan metabolism.

SUMMARY

1. It has been found that in a large proportion of cases the febrile human excretes hydroxykynurenine. This excretion is, in particular, unrelated to tuberculosis or diabetes. It is attributed to a high rate of breakdown of body protein.

2. In states associated with weight loss but not accompanied by fever, hydroxykynurenine excretion could not be detected.

3. The normal human taking an excess of tryptophan by mouth excretes much kynurenine, but no, or negligible, hydroxykynurenine.

4. Possible reasons for this difference in excretory patterns are discussed.

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The Metabolism of Progesterone by Animal Tissues *in vitro*

1. FACTORS INFLUENCING THE METABOLISM OF PROGESTERONE BY RAT LIVER AND THE INVESTIGATION OF THE PRODUCTS OF METABOLISM

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Table 1 lists the steroids closely related to progesterone which have been isolated from human pregnancy urine (see Samuels & West, 1952, for references) and which are therefore assumed to be metabolites of the hormone. By *in vivo* studies, only three of these steroids have been shown conclusively to be derived from progesterone, namely, pregnane-3α:20α-diol (pregnanediol) (Venning & Browne, 1937), pregnan-3α-ol-20-one (pregnanolone) (Dorfman, Ross & Shipley. 1948) and 5α-pregnane-3α:20α-diol (Kyle & Marrian, 1951). In spite of indications from *in vivo* studies that the liver is involved in progesterone metabolism (see Samuels & West, 1952, for references), Zondek (1941) and Engel (1944) were unable to demonstrate inactivation of the hormone by this tissue *in vitro*. However, using more sensitive chemical methods, Samuels (1950) showed that the $\alpha\beta$-unsaturated ketonic group of progesterone underwent some change when incubated with rat-liver mince.

Table 1. *Human urinary steroids related to progesterone*

Pregnane-3:20-dione
5α-Pregnane-3:20-dione
Pregnan-3α-ol-20-one
5α-Pregnan-3α-ol-20-one
5α-Pregnan-3β-ol-20-one
Pregnane-3α:20α-diol
Pregnane-3β:20α-diol
5α-Pregnane-3α:20α-diol
5α-Pregnane-3β:20α-diol
Pregnan-3α-ol

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It was, therefore, decided that further investigation of the *in vitro* metabolism of progesterone should be undertaken. For this purpose a method was developed for the determination of progesterone extracted from incubated tissue preparations. The method, which is based on the partition-chromatographic method of Butt, Morris, Morris & Williams (1951) for the determination of progesterone in blood, is more sensitive and less tedious than those employed by others (cf. Samuels, McCaulay & Sellers, 1947; Hayano & Dorfman, 1953). Contrary to the finding of Wiswell & Samuels (1953) it has been shown that the metabolism of progesterone by rat-liver 'homogenate' is increased by addition of diphosphopyridine nucleotide (DPN), but in confirmation of the finding of these authors, it has been demonstrated that metabolism is increased in the presence of citrate and is not inhibited under anaerobic conditions.

In a preliminary investigation of the products of metabolism, 5α-pregnane-3:20-dione and 5α-pregnan-3α-ol-20-one have been isolated.

EXPERIMENTAL

Materials and methods

Adenosine triphosphoric acid (ATP) and adenylic acid (AMP) obtained from Light and Co. were used without further purification. DPN was prepared in the laboratory by the method of LePage (1949) and its purity as determined spectrophotometrically (LePage, 1947) was 37%. The standard medium used throughout this investigation was the calcium-free phosphate saline (pH 7·4) of Krebs & Eggleston (1940) (subsequently referred to as 'phosphate saline'). Progesterone (Organon Laboratories) was purified by two crystallizations from hexane and one from aqueous ethanol and had m.p. 121–121·5°.

All melting points were determined on an improvised hot-stage apparatus of the Kofler type (Klyne & Rankeillor, 1947) and are corrected for the emergent stem.

Specific rotations were determined for the *D* line using a 0·5 dm. microtube. The errors were calculated as described by Klyne & Paterson (1948).

In partition chromatography, Celite 535 (Johns-Manville Co.) was used as the supporting phase. It was purified before use by heating at 400° for 8 hr., by treatment with 10N-HCl for 12 hr. at room temperature followed by intensive washing with water until free from acid, and finally by air-drying at 130° for at least 12 hr.

Methanol and ethanol were refluxed with solid KOH and twice distilled. Hexane was washed with conc. H_2SO_4, dried over metallic Na and distilled. Benzene (A.R.) and ether (A.R.) were dried over Na and redistilled; $CHCl_3$ was freshly distilled before use. Acid-free acetone was dried by distillation from anhydrous K_2CO_3. In adsorption chromatography, alumina (Peter Spence and Co.) activated by heating at 100° *in vacuo* (activity II, Brockmann & Schodder 1941) was used throughout. The amount of alumina employed was 30 times the weight of material to be chromatographed and columns of length 10 times the diameter were used.

Liver preparations. Experimental animals were female rats of the Wistar strain about 6 months old. They were killed by dislocation of vertebrae in the neck followed by exsanguination. The livers were rapidly removed and placed in chilled 0·15M-KCl. Slices of about 0·3 mm. thickness were prepared as described by Grant & Marrian (1950). Samples were weighed on a torsion balance to the nearest 2 mg.

Suspensions of tissue were prepared in tissue grinders made in the laboratory which had a glass outer tube and a motor-driven, piston-type, nylon pestle about 3·0 cm. long and 1·5 cm. in diameter. The tissue suspensions or 'homogenates', so obtained were found to be uniformly acellular (cf. Brendler, 1951). The tissue (2·5 g.) was ground with 6 ml. ice-cold 0·15M-KCl (unless otherwise stated) for about 1 min. The final suspension or 'homogenate' was diluted to 10 ml. with 0·15M-KCl and was kept at 0° until pipetted into incubation flasks.

Method employed for the determination of progesterone

For the purpose of the present investigation it was necessary to devise a method for the quantitative determination of about 0·1–0·5 mg. progesterone in the presence of 250 mg. tissue as slices or pulp. The extraction procedure was most easily carried out in glass-stoppered test tubes (Quickfit and Quartz, standard B19 ground-glass joint, capacity 30 ml.). Transferences to and from the extraction tubes were made by means of an adaptor constructed from a B19 joint (Fig. 1) into which could be fitted: (*a*) a Hirsch funnel, (*b*) suction tube *A* having a straight tip for transferences to the extraction tubes (see below), and (*c*) suction tube *B* having a U-tip for transferences from the extraction tube (Fig. 1).

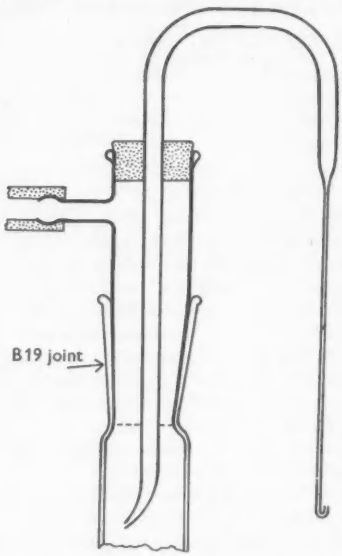

Fig. 1. Adaptor and U-tipped suction tube *B* for transferences from extraction tubes.

Protein precipitation. At the conclusion of the incubation period, 15 ml. acetone were added to each flask and after having been chilled to −20° the contents were filtered with suction through Whatman no. 1 paper into 100 ml. flasks with the aid of the adapter fitted with a Hirsch funnel. The precipitate and paper were transferred to the original flask and washed with 3 × 10 ml. hot acetone; each time before filtration the flasks were chilled to −20°. When slices were used, after two such washings, the slices were ground with 10 ml. acetone and the resultant powder was extracted with 2 × 10 ml. acetone, with chilling to −20° before filtration. The combine filtrates were concentrated to about 3 ml. aqueous residue by removing the acetone in a stream of air at 45°.

Extraction of aqueous residue. To the aqueous residue were added 6 ml. hexane:chloroform (9:1, v/v) and the mixture was refluxed for 5 min. After being cooled, the mixture was transferred, by means of suction tube *A*, to the extraction tube and the flask rinsed with 3 × 5 ml. hexane:chloroform, 2 × 2 ml. water and finally with 2 ml. hexane:chloroform, each rinsing being transferred to the extraction tube. After being centrifuged to separate the emulsions, the upper phase was transferred to the original 100 ml. flask by means of suction tube *B* and the aqueous phase extracted with 2 × 20 ml. hexane:chloroform. The combined extracts were evaporated to dryness in a stream of air at 45°, removal of the last traces of water being facilitated by addition of a little ethanol. The residues were finally dried for at least 1 hr. *in vacuo* over P_2O_5.

Separation of progesterone from lipid extract. Separation of progesterone from the lipid extract was achieved by means of partition chromatography. Equilibration of solvents and preparation and running of columns were carried out in a room thermostatically controlled within the range 16–18° and usually at 17·5±0·5°.

Columns (10 cm. long, 1·0 cm. diameter) were packed in the manner described by Butt *et al.* (1951) in glass tubes about 18 cm. long, one end being fitted with a B 14 standard joint and the other being flat glass with small holes (0·5 mm. diameter) regularly spaced. The first pad, packed effectively, sealed the holes at the base of the tube and no leakage of Celite occurred during the running of the columns. The system methanol:water (7·5:2·5, v/v) (stationary phase) and hexane (mobile phase), having 1·0 ml. stationary phase/g. Celite, was found to be suitable for the separation of progesterone from other material absorbing at 240 mμ. Preliminary experiments showed that progesterone was eluted from columns of these dimensions between 13 and 20 ml.

To each extract obtained as above were added 5·0 ml. mobile phase. Since the incubation and extraction procedures required 1 day to complete, it was convenient to leave the tightly stoppered flasks overnight in order to ensure complete solution of the extract. The columns were packed and 10 ml. mobile phase allowed to run through them. After pouring off the excess of mobile phase from the top of the column, 0·50 ml. extract was added and the walls of the column were rinsed with 3 × 0·5 ml. mobile phase, each washing being allowed to disappear just below the surface of the Celite before the next was applied. During the application of the solution and the washing of the column, the eluate was collected and formed part of the first fraction. About 4·0 ml. mobile phase were then added, care being taken not to disturb the surface of the Celite. A reservoir with a capillary outlet was fitted to the column and the rate of flow checked over a period of 10 min. When the flow rate was outside the limits, 8–12 ml./hr., the column was rejected and a fresh one prepared. The first 10 ml. eluate were rejected and the next 12 ml. (progesterone fraction) collected for analysis. The solvent was removed in a stream of air and 5 ml. absolute ethanol were added to the dry residue. The amount of progesterone present in the solution was determined by measuring the optical density of the solution at 240 mμ. with a Unicam S.P. 500 Spectrophotometer in a 1·0 cm. cell.

As a test of the accuracy and specificity of the method, a series of 'recovery' experiments was carried out in which progesterone was added to incubated liver preparations. These experiments were carried out as follows.

Known amounts of progesterone in propylene glycol solution were added to 250 mg. samples of tissue as slices or homogenate which had been incubated at 37° for 1 hr. Acetone was immediately added and the mixture processed as described above. In 'blank' experiments, progesterone solution was replaced by propylene glycol. The results of these experiments shown in Table 2 indicate that the method is satisfactory for the determination of as little as 0·1 mg. progesterone added to 250 mg. tissue.

The incubation of progesterone with rat-liver preparations

Each experiment was conducted in four parts.

(1) *Incubation experiments.* Duplicate 250 mg. portions of slices were added to 25 ml. conical flasks standing on ice and containing 1·0 ml. 0·15 M-KCl and 2·0 ml. 'phosphate saline'. In the case of 'homogenate', duplicate 1·0 ml. volumes equivalent to 250 mg. tissue were added to 2·0 ml.

Table 2. *Recovery of progesterone added to incubated rat-liver preparations*

(In seven 'blank' experiments, the following quantities of apparent progesterone were recovered: 0·014, 0·016, 0·015, 0·014, 0·015, 0·016 and 0·016 mg. The mean 'blank' value (0·015 mg.) was used to correct the values given below.)

Preparation	Progesterone added (mg.)	No. of results	Progesterone recovered (corrected for 'blank') (%)	Range (%)
Slices (250 mg.)	0·5	8	95±1	92–97
	0·2	4	90±1	89–91
'Homogenate'	0·5	18	97±1	90–100
	0·2	5	95±2	91–98
	0·1	5	89±2	87–95

'phosphate saline'. Unless otherwise stated, additions to the reaction mixture were made with the substance dissolved in 'phosphate saline' and maintaining the volume at 3 ml. by a corresponding reduction in the volume of 'phosphate saline' originally added. A solution of 0·5 mg. progesterone in 0·050 ml. propylene glycol was added to each flask by means of an 'Agla' micrometer syringe (Burroughs, Wellcome and Co.), and when all flasks had been prepared they were shaken for 1 hr. in air (unless otherwise stated) at 37°.

(2) *Steroid recovery controls* ('*controls*'). In these, progesterone was incubated in 'phosphate saline' (with added substances when used) and at the end of the incubation period, incubated tissue was added and the contents of the flask were immediately processed.

(3) *Controls without added steroid* ('*blanks*'). These were introduced in order to ensure that the incubation of liver with propylene glycol alone did not result in production of material absorbing at 240 mμ. and which was not removed by the purification procedure employed. The procedure was as described in (1), except that progesterone solution was replaced by propylene glycol. The values of apparent progesterone obtained from 'blank' experiments were invariably low and consistent. They were therefore not carried out routinely but were checked occasionally.

(4) *Determination of progesterone content of propylene glycol solution.* This determination was carried out on duplicate volumes of the solution added to incubation and control vessels by pipetting 0·010 ml. volumes into tubes containing 3 ml. ethanol and evaporating to dryness. The optical density of the residue was determined at the same time as the residues obtained from the incubation experiment.

It may be seen from the results of these experiments shown in Fig. 2 that progesterone disappears on incubation with tissue slices and cell-free suspensions. The lower activity of cell-free suspensions in metabolizing progesterone is evident from the fact that with these preparations about 80% of the progesterone originally present was recovered, as compared with the recovery of only about 64% progesterone incubated under the same conditions with slices from the same liver. A similar loss of activity on mincing liver has been observed in metabolic studies with oestrogens (Coppedge, Segaloff & Sarrett, 1950), with testosterone (Sweat & Samuels, 1948) and with pregnanediol (Grant, 1952), and has been attributed by these workers to the rapid destruction of DPN in preparations of broken cells from liver and other tissues (Mann & Quastel, 1941). The pH of 7·4 used in the various liver-steroid metabolism experiments reported by others and used in the present investigation is close to the optima of 7·2 (Spaulding & Graham, 1947) and 7·5 (Handler & Klein, 1942) which have been reported for the nucleosidase concerned in the destruction of DPN. This destruction is, however, inhibited by nicotinamide (Mann & Quastel, 1941; Handler & Klein, 1942).

Effect of DPN and nicotinamide. In order to determine whether the reduced activity of 'homogenate' could be accounted for by the destruction of endogenous DPN, the experiment illustrated in Fig. 3 was carried out.

It may be observed that there is a decrease in metabolism when 'homogenate' is pre-incubated (bar *C*) compared with 'homogenate' not pre-incubated (bar *B*). This decrease is overcome by adding nicotinamide before (bar *D*) or DPN after (bar *G*), pre-incubation. It is not fully prevented by adding DPN before pre-incubation (bar *F*). The failure of nicotinamide added after pre-incubation to affect markedly the degree of metabolism (bar *E*) shows that

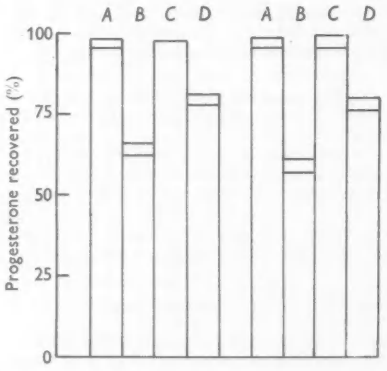

Fig. 2. Incubation of progesterone with rat-liver slices and suspensions of disintegrated tissue ('homogenates'). Reaction mixture: about 0·5 mg. progesterone, 2·0 ml. 'phosphate saline', 1 ml. 0·15 M-KCl + 250 mg. liver as slices or 'homogenate'. Progesterone recovered, *A*, from 'controls' (slices); *B*, after incubation with slices; *C*, from 'controls' ('homogenate'); *D*, after incubation with 'homogenate'. In this type of figure, double lines at ends of bars indicate duplicates; single lines coincident duplicates.

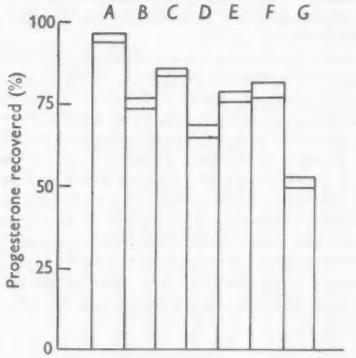

Fig. 3. Incubation of progesterone with suspensions of disintegrated rat-liver tissue. Reaction mixture (RM): about 0·5 mg. progesterone, 2·0 ml. 'phosphate saline' + 1 ml. 'homogenate'. Progesterone recovered, *A*, from 'controls'; after incubation of the following: *B*, RM; *C*, RM using 'homogenate' pre-incubated 0·5 hr.; *D*, RM using 'homogenate' pre-incubated 0·5 hr. with nicotinamide (final concn. 40 mM); *E*, RM using 'homogenate' pre-incubated 0·5 hr. with nicotinamide (final concn. 40 mM) added after pre-incubation; *F*, RM using 'homogenate' pre-incubated 0·5 hr. with DPN (final concn. 3·0 mM) added before pre-incubation; *G*, RM using 'homogenate' pre-incubated 0·5 hr. with DPN (final concn. 3·0 mM) added after pre-incubation.

nicotinamide itself is not responsible for increased progesterone metabolism, but that it exerts its effect by protection of endogenous DPN. The observation that preincubation without added DPN or nicotinamide (bar *C*) did not completely inactivate the 'homogenate' suggests that alternative pathways exist for the metabolism of progesterone and that one or more is not sensitive to DPN.

The effect of various concentrations of DPN and nicotinamide on the metabolism of progesterone by rat-liver 'homogenates' is shown in Fig. 4. It appears that relatively high concentrations of DPN are required to obtain a degree of metabolism comparable with that obtained with the same weight of tissue as slices (Fig. 2). The DPN preparation was only 37% pure, and it is possible that it contained impurity capable of inhibiting the enzymes responsible for progesterone metabolism. Adenylic acid is the chief impurity in the DPN as prepared (LePage, 1949), but when this substance replaced DPN in the reaction mixture in a concentration of 5·0 mM, it had no effect on the activity of the cell-free suspension in metabolizing progesterone. It may be seen from Fig. 4 that an optimum effect was obtained using 0·7 mM DPN and 40 mM nicotinamide.

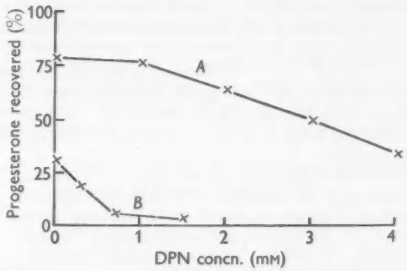

Fig. 4. Incubation of progesterone with suspensions of distintegrated rat-liver tissue. Effect of varying concentrations of DPN. *A*, after incubation with 'homogenate' prepared in 0·15 M-KCl; *B*, after incubation with 'homogenate' prepared in 0·15 M-KCl – 0·12 M nicotinamide.

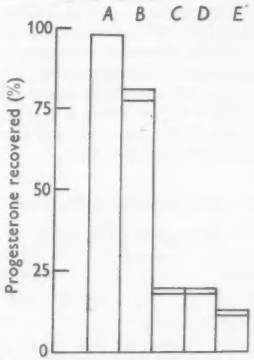

Fig. 5. Incubation of progesterone with suspensions of disintegrated rat-liver tissue; influence of citrate on progesterone metabolism. Reaction mixture (RM): about 0·5 mg. progesterone, 2·0 ml. 'phosphate saline' +1 ml. 'homogenate' prepared in 0·15 M-KCl. Progesterone recovered: *A*, from 'controls'; *B*, after incubation of RM; after incubation of RM with citrate; *C*, 1·0 mM citrate, *D*, 2·0 mM citrate and *E*, 3·0 mM citrate.

Effect of other added substances. Fig. 5 illustrates the effect of varying concentrations of citrate on the metabolism of progesterone by rat-liver 'homogenate'. It can be seen that the effect of citrate is almost maximal at a concentration of 1·0 mM. The following substances were tested in the standard reaction mixture and were found to be without effect on the metabolism of progesterone; ATP (0·5 mM), fumarate (1·0 mM), α-oxoglutarate (1·0 mM), malate (1·0 mM), succinate (1·0 mM).

Effect of anaerobic conditions. Replacement of air in the reaction vessels by nitrogen or evacuation of the reaction vessels resulted in no change in the amount of progesterone metabolized (Fig. 6).

Investigation of the products of metabolism

It was considered that incubation of 300 mg. of progesterone with rat liver under optimum conditions would be a convenient scale for the isolation of the major metabolites. At a tissue:steroid ratio of 500:1, this amount of progesterone required 150 g. liver (obtainable from about thirty rats). Since it was intended to include a 'control' (steroid added after incubation) and a 'blank' (no added steroid) the large scale experiment was conducted in two parts. In the first part, 150 mg. progesterone were incubated with tissue suspension and a 'control' treated in identical fashion was run at the same time. The tissue suspension was prepared in small batches as described above. The Waring Blendor, with high-speed rotating blades, was not used for the preparation of the larger quantities of tissue suspension required since its use results in excessive frothing, the temperature is difficult to control, and it has been reported by Stern & Bird (1949) and Lambden (1950) that it is capable of inactivating certain enzymes.

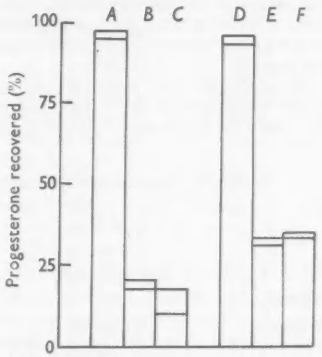

Fig. 6. Incubation of progesterone with suspensions of disintegrated rat-liver tissue. Reaction mixture: about 0·5 mg. progesterone, 2·0 ml. 'phosphate saline' +1 ml. 'homogenate' prepared in 0·15 M-KCl containing 0·12 M nicotinamide. Progesterone recovered; *A* and *D*, from 'controls': after incubation; *B*, in air; *C*, in nitrogen; *E*, in open Thunberg tube; *F*, in evacuated Thunberg tube.

In the second part, a further 150 mg. progesterone were incubated and a 'blank' was run at the same time.

It was convenient to conduct the incubations in 100 ml. conical flasks; thirty of these were prepared. To each were added 20 ml. chilled 'phosphate saline' and 10 ml. tissue suspension. (The 'homogenizing' was carried out in 0·15 M-KCl containing nicotinamide in sufficient quantity to provide a concentration of 80 mM in the final reaction mixture.) A propylene glycol solution of progesterone (10 mg./ml.) was warmed to 37° to reduce the viscosity of the solvent, and 1·0 ml. volumes were pipetted into each of the 'incubation' flasks. Pure solvent (1·0 ml.) was added to the 'control' and 'blank' flasks. The flasks were not stoppered but were shaken vigorously during the incubation period of 2 hr. After incubation, 'incubation', 'control' and 'blank' experiments were treated in the same manner except that in the case of the 'control', 150 mg. of solid progesterone were added after addition of acetone.

At the conclusion of the incubation period, the contents of the flasks were emptied into a large beaker. Each flask was rinsed with 50 ml. portions of hot acetone until a total of 2 l. acetone had been used, and the washings were added to the beaker. After being vigorously stirred, the mixture was allowed to stand overnight at −20°. The yellow supernatant was filtered and the precipitate returned to the beaker and washed twice with 1 l. volumes of acetone previously chilled to −20°. The bulk of the acetone was removed from the combined filtrates by distillation under reduced pressure (in this and subsequent distillations, the bath temperature was kept below 60°). After dilution with 250 ml. water, the aqueous residue was saturated with NaCl and the yellow oil which separated was extracted with 4 × 250 ml. ethyl acetate. The ester extract was washed successively with 80 ml. volumes water, 0·2 M-$NaHCO_3$, 0·2 N-HCl and finally with water until the washings were neutral. Washing was carried out rapidly so that the ester was in contact with acid or alkali for the shortest possible time. The first water and $NaHCO_3$ washes were combined and back-extracted with 200 ml. ethyl acetate and the same 200 ml. was used to back-extract the HCl and water washes individually. After being dried over anhydrous Na_2SO_4, the combined ester extracts were evaporated to dryness by distillation *in vacuo*.

The resultant brown gum was taken up in 300 ml. methanol and 100 ml. hexane; 100 ml. water were added and two layers separated, the hexane layer being appreciably yellow. The mixture was transferred to a separating funnel and the distillation flask rinsed with 100 ml. 75% (v/v) aqueous methanol and 30 ml. hexane, the rinsings being transferred to the separating funnel. The aqueous methanol was run off and the hexane extracted with 2 × 100 ml. aqueous methanol and the combined extracts washed with 50 ml. hexane. The bulk of the methanol was removed by distillation *in vacuo*, 150 ml. water were added and the aqueous residue was extracted with 2 × 500 ml. $CHCl_3$. The chloroform extract was washed with 100 ml. N-NaOH and then with water until the washings were neutral. After being dried over anhydrous Na_2SO_4, the $CHCl_3$ was removed by distillation *in vacuo*. The weights of the extracts obtained at this stage were: 'incubations', 0·249 g. and 0·223 g.; 'control', 0·30 g. and 'blank', 0·436 g.

The extracts were then subjected to an eight-stage, discrete countercurrent distribution (Craig & Craig, 1950), carried out in separating funnels, using the solvent system methanol:water (80:20, v/v) and benzene:hexane (80:20, v/v) in which the partition coefficient (K) of progesterone was found to be 0·25 $\left(K = \frac{\text{concn. in aqueous methanol}}{\text{concn. in benzene:hexane}}\right)$. A high degree of purification was obtained in this way; the residues from funnels 0–4 of 'incubation' and 'control' extracts were white and semi-crystalline but melting points were not sharp.

The residues from funnels 0–4 were combined and chromatographed on alumina. The material dissolved in a minimum volume of 50:50 (v/v) benzene:hexane was applied to the column, and the following fractions were collected: 5 × 10 ml. benzene:hexane (50:50, v/v); 10 × 10 ml. benzene; 5 × 10 ml. benzene:ether (95:5, v/v); 5 × 10 ml. benzene:ethanol (80:20, v/v) and 25 ml. ethanol.

Isolation of 5α-pregnane-3:20-dione. The benzene:hexane and benzene fractions from the 'control' and 'blank' chromatograms yielded only a small amount of gum, but the benzene fractions from the 'incubations' yielded a total of 40 mg. semi-crystalline material. After two crystallizations from acetone, this material yielded 30 mg. shimmering, white plates, m.p. 199–200° (substance X). A comparison of the properties of this substance with those of a sample of authentic 5α-pregnane-3:20-dione (prepared by reduction of pregn-5-en-3β-ol-20-one with H_2 in presence of Pt followed by oxidation with CrO_3 in acetic acid solution (Marker, Kamm & McGrew, 1937), provided proof that substance X was 5α-pregnane-3:20-dione (Table 3).

Table 3. *Comparison of properties of substance X and of 5α-pregnane-3:20-dione*

		5α-Pregnane-3:20-dione	
	'X'	Found	Reported
M.p.	199–200°	199–200·5°	200–201° (*a*)
Mixed m.p.	198·5–199·5°		
C, H Analysis	Found C: 79·5, 79·5 H: 10·5, 10·4	C: 79·6, 79·5 H: 10·3, 10·3	Calc. for $C_{21}H_{32}O_2$: C, 79·7 H, 10·2
$[\alpha]_D$ in EtOH	+110±1·5° at 19·5° (*c*, 0·537)	+108±2·8° at 19° (*c*, 0·5897)	+117±4° at 24° (*a*) (*c*, 0·255)
Disemicarbazone			
M.p.	>300°	>300°	>300° (*b*)
Mixed m.p.	>300°		

(*a*) Lieberman, Dobriner, Hill, Fieser & Rhoads (1948). (*b*) Kyle & Marrian (1951).

Isolation of progesterone. The ether:benzene fractions from the 'control', after recrystallization from aqueous ethanol yielded 84 mg. progesterone, while from the 'incubation' 12 mg. of this steroid were obtained. Progesterone was identified by m.p., mixed m.p. and u.v. absorption spectrum. No crystalline material was obtained from the 'blank'.

Isolation of 5α-pregnan-3α-ol-20-one. Only the 'incubation' yielded any appreciable material in the ethanol:benzene fractions. The residues were waxy and melted over a wide range; they were therefore chromatographed on alumina a second time. The combined residues were added in benzene and the column eluted with the following series of solvents, 5 ml. fractions being collected: 30 ml. benzene; 35 ml. ether:benzene (1:9, v/v); 15 ml. ether:benzene (5:5, v/v); 20 ml. ether:benzene (9:1, v/v) and finally with 25 ml. ethanol. Two main fractions were obtained; the 1:9 ether:benzene fractions yielded 25 mg. of a semi-crystalline solid, m.p. 165–172° (substance Y) and the 5:5 ether:benzene fractions yielded 22 mg. of a colourless gum which turned white on addition of hexane (substance Z).

Substance Y was recrystallized from aqueous methanol and yielded 20·5 mg. of thin plates, m.p. 173–175°. It gave no precipitate with digitonin and, mixed with 5α-pregnan-3β-ol-20-one (m.p. 193–195°; kindly supplied by Dr J. Y. F. Paterson), the m.p. was 152–167°. A comparison of the properties of this substance with those reported for 5α-pregnan-3α-ol-20-one and the finding that admixture of the acetate with an authentic sample resulted in no depression of the melting point, provided proof that substance Y was 5α-pregnan-3α-ol-20-one (Table 4).

Table 4. *Comparison of properties of substance 'Y' and of 5α-pregnan-3α-ol-20-one*

	'Y'	5α-Pregnanolone*
M.p.	173–175°	174–175°
C, H Analysis	Found C, 79·4, 79·0 H, 10·9, 10·9	Calc. for $C_{21}H_{34}O_2$ C, 79·2 H, 10·8
$[\alpha]_D$ in EtOH	+99·0±1° at 21° (*c*, 0·524)	+96·6±1° at 25° (*c*, 0·755)
Acetate		
M.p.	141–143°	140–142°
Mixed m.p.	140–142°†	
C, H Analysis	Found C, 76·0 H, 9·9	Calc. for $C_{23}H_{36}O_3$ C, 76·6 H, 10·1

* Lieberman *et al.* (1948).
† Sample supplied by Dr H. Hirschmann, Lakeside Hospital, Cleveland.

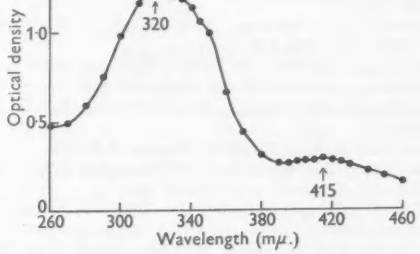

Fig. 7. Absorption spectrum of H_2SO_4 chromogen of substance Z; 320 μg. substance Z + 5·0 ml. conc. H_2SO_4 in 1·0 cm. cell.

Investigation of substance Z. Recrystallization of this substance was attempted from a number of solvents but without success, and so it was rechromatographed; no separation was obtained. The material was eluted from activity II alumina by ethanol:benzene (1:99, v/v) but not by benzene. Fractional crystallization from aqueous acetone yielded three specimens of silky, white needles, m.p.'s 123–130°, 121–130° and 105–124°. Admixture with androstane-3:17-dione (m.p. 132–134°; prepared by oxidation of androsterone with CrO_3) depressed the m.p. in each case. The three specimens were combined and recrystallized from aqueous acetone. The resultant crystals (2·5 mg., m.p. 124–130°) yielded no formaldehyde on oxidation with HIO_4, gave no Zimmermann reaction, had no significant absorption in the ultraviolet region and gave no precipitate with digitonin. With conc. H_2SO_4 a faint yellow colour was obtained at a concn. of 64 μg./ml. conc. H_2SO_4. The absorption spectrum of this solution is shown in Fig. 7.

The mother liquors from the fractional crystallizations were combined and the residue (12 mg.) separated into α and β fractions by means of digitonin. The α fraction (6·7 mg.) could not be crystallized from a variety of solvents, and so was benzoylated and chromatographed on alumina (Brooks, Klyne & Miller, 1953). Recrystallization from $CHCl_3$ gave 1·8 mg. fine white needles which melted at 67°, solidified on further heating, at 110°, and finally melted at 125–126°. Brooks *et al.* (1953) give m.p. 131–133° for 5β-pregnane-3α:20α-diol dibenzoate but do not report a double m.p. Attempts to crystallize the β fraction failed. It is intended to investigate the nature of substance Z more fully by carrying out an isolation experiment on a larger scale.

DISCUSSION

The evidence presented in this paper shows that diphosphopyridine nucleotide and citrate increase the activity of the enzyme system or systems involved in the *in vitro* metabolism of progesterone by rat-liver 'homogenate'. The isolation of two 5α-pregnane derivatives provides evidence that the observed loss of progesterone on incubation of the steroid with rat-liver 'homogenate' is the result, at least in part, of a reductive process. At this stage of the work, however, it would be unwise to attempt to discuss the possible biochemical significance of the enzymic systems involved.

It is of interest that the metabolic reduction of the double bond in ring A was found to be sterically directed to yield only 5α-pregnane derivatives. This finding is in agreement with that of Schneider (1952) who isolated 5α-pregnane derivatives but no pregnane (5β) derivatives after incubation of 11-deoxycorticosterone with rat-liver slices and with that of Caspi, Levy & Hechter (1953) who demonstrated the formation of 5α-pregnane derivatives when cortisone was metabolically reduced by perfusion through rat liver. In the present work,

however, only one of the two possible C-3 epimeric alcohols was obtained, namely, 5α-pregnan-3α-ol-20-one (5α-pregnanolone), whereas Schneider (1952) and Caspi *et al.* (1953) demonstrated that both epimers were formed in their experiments. In the case of deoxycorticosterone (pregn-4-en-21-ol-3:20-dione), the main reduction product was 5α-pregnane-3β:21-diol-20-one and only a small quantity of the 3α epimer was obtained (Schneider, 1952). It would appear that the nature of the C-17 side-chain influences the steric course of the reduction of the C-3 ketonic group of non-benzenoid steroids.

The isolation of 5α-pregnane-3:20-dione as a major metabolite supports the findings of Riegel, Hartop & Kittinger (1950) who demonstrated that after injection of [21-^{14}C]progesterone into rats, most of the radioactivity was found in the faeces in a ketonic, non-alcoholic, neutral steroid which was not progesterone. The failure to isolate any 'pregnanediols' in the present investigation does not necessarily indicate that such substances were not formed. It is not known whether the rat excretes 'pregnanediols' normally or after progesterone administration, but the possibility of detecting such substances is reduced in view of the fact that rat-liver 'homogenate' is able to metabolize pregnane-3α:20α-diol *in vitro* to pregnan-3α-ol-20-one (Grant, 1952).

In the large-scale experiments now reported, only 28% of the progesterone added could be accounted for in terms of the two metabolites and unchanged progesterone isolated. While some of this loss is no doubt manipulative (only 56% of added progesterone was recovered from the 'control'), it is apparent that it reflects enzymic degradation of progesterone to substances not recoverable by the isolation techniques employed. The nature of substance Z requires further investigation.

SUMMARY

1. A method is described for the determination of small quantities of progesterone in incubated tissue preparations.

2. The application of the method to the study of the *in vitro* metabolism of progesterone by rat-liver slices and suspensions of disintegrated tissue ('homogenates') has indicated that this tissue contains an enzyme or enzymes which metabolize this steroid. The metabolism is increased by the addition of either diphosphopyridine nucleotide or nicotinamide or both, and of citrate to the reaction mixture; it is not inhibited under anaerobic conditions.

3. In a large-scale experiment, 28%, of the progesterone incubated with rat-liver 'homogenate' (with added nicotinamide) could be accounted for by the isolation of 5α-pregnane-3:20-dione and 5α-pregnan-3α-ol-20-one and of unchanged progesterone.

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The Effect of *p*-Nitrophenol on the Pasteur Reaction and on Aerobic Phosphorylation in Suspensions of the Mammary Gland

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It is well known that certain inhibitors of the 'Pasteur effect', in particular the nitrophenols, are also inhibitors of oxidative phosphorylation. The Pasteur effect and oxidative phosphorylation have been studied extensively in suspensions of intact yeast cell (Seits & Engelhardt, 1949; Lynen & Koenigsberger, 1951). Recently, Meyerhof & Fiala (1950) demonstrated the two phenomena and their simultaneous inhibition by *p*-nitrophenol (*p*NP) in a dried-yeast preparation, and concluded that the Pasteur effect results from a phosphorylating step.

In work with animal tissues, the Pasteur effect and aerobic phosphorylation have so far been studied in different types of preparation, the former in tissue slices, the latter in broken-cell preparations, and conclusions regarding the common effect of inhibitors on the two phenomena have been made by analogy, a procedure which is open to criticism (see Judah & Williams-Ashman, 1951).

The difficulty of obtaining broken-cell preparations which exhibit a Pasteur effect has led to the view that this phenomenon is dependent to some degree on the structural integrity of the tissue (see Dickens, 1951). In the present work a marked Pasteur effect and vigorous aerobic phosphorylation are shown to occur in cell-free suspensions of the lactating mammary gland of the guinea pig. Both phenomena are sensitive to the action of *p*NP which supports the widely held view that they are closely connected. The results presented extend the findings of Meyerhof & Fiala (1950) to animal tissues. A preliminary account of part of this work has been given to the Biochemical Society (Terner, 1953*a*).

EXPERIMENTAL

Material. The mammary glands of lactating rats or guinea pigs were removed after the death of the animals and placed in ice-cold isotonic KCl. The tissue was passed through a Latapie mincer and washed three times in cold KCl and strained on muslin. The minced tissue was then ground in an all-glass homogenizer (Potter & Elvejehm, 1936) in a medium of isotonic KCl containing 0·024 M-$KHCO_3$ and 0·02 M nicotinamide. To prepare brain suspensions, the brains of two guinea pigs were trimmed to remove as much white matter as possible and were ground in a glass homogenizer in the solution described above, without preliminary washing. The suspensions were further diluted with isotonic KCl to give in most cases a 1 in 5 suspension (dry wt. 40–50 mg./ml.).

Although the mammary suspensions contained shreds of connective tissue, they were, on microscopic examination, found to contain nuclei but to be free from unbroken cells. Brain suspensions, however, contained relatively large numbers of whole cells.

Reaction mixture. The basal medium contained in addition to isotonic KCl: $MgCl_2$, 0·005–0·01 M; nicotinamide, 0·02 M; cozymase, $1{\cdot}25–2{\cdot}5 \times 10^{-4}$ M; adenosine triphosphate (ATP), 0·001 M; phosphate buffer, pH 7·4, 0·0025 M; and either glycylglycine buffer, pH 7·4, 0·02 M (all aerobic and some anaerobic experiments), or $KHCO_3$, 0·024 M (anaerobic experiments). Further additions were made as stated in the text and in the tables. In aerobic experiments the gas was air. Cytochrome *c* was not a factor limiting the rate of respiration of mammary suspensions under the experimental conditions described and was only added where stated. When anaerobic conditions were required, a stick of yellow phosphorus was placed in the centre-well and the manometer was gassed with 5% CO_2+95% N_2, or with 100% N_2.

The suspension (1 ml.) and hexokinase (when added) were placed in the side-arm of the Warburg flask and were mixed with the medium in the main compartment immediately before the vessel was placed in the thermostat at 37°. The volume of the complete reaction mixture was 4·0 ml. Readings were begun after 10 min. equilibration and were continued at intervals of 5 min. All manometric data have been corrected by extrapolation to include the equilibration period.

After incubation the vessels were cooled in iced water and 3 ml. 20% trichloroacetic acid or 1·5 N perchloric acid (Neuberg, Strauss & Lipkin, 1944) were added. Several zero-time control flasks, containing all the constituents in the various combinations used, were deproteinized at the moment of the start of the incubation period. The protein-free filtrates were either analysed immediately or after neutralization to pH 6·5–6·6 and storage at −17°.

Analytical methods. Inorganic phosphate was estimated by a modification of the method of Berenblum & Chain (1938) described by Weil-Malherbe & Green (1951); lactic acid by the method of Barker & Summerson (1941); fructose 1:6-diphosphate (HDP) by the determination of inorganic phosphate liberated after 3 min. hydrolysis in 0·2 N alkali at 100° (Kiessling, 1938) and also by the estimation of fructose according to Roe (1934).

Reagents and preparations. The potassium salts of HDP and of ATP were prepared from commercial barium salts. Cozymase (85% pure) was a product of Schwarz Laboratories Inc., New York, U.S.A. Cytochrome *c* was obtained commercially as a dried preparation.

Hexokinase was prepared from baker's yeast according to Meyerhof (1927). Before each experiment 20 mg. of the powder were ground in 2 ml. distilled water and the insoluble matter was centrifuged off; 0·1 ml. of the extract contained about 9 hexokinase units as defined by Berger, Slein, Colowick & Cori (1946). An extract of rabbit-muscle acetone powder was prepared as described by Green, Needham & Dewan (1937). Crystalline potassium pyruvate was prepared according to Korkes, del Campillo, Gunsalus & Ochoa (1951). A sample of *p*NP was purified by repeated crystallization from hot water. Sodium fluoroacetate was kindly given by Sir Rudolph Peters. Insulin free from glycogenolytic factor was a gift to Dr Folley from Dr K. Hallas Møller of Novo Terapeutisk Laboratorium, Copenhagen.

Units. All metabolic quotients are expressed in the Q notation (μl./mg. dry wt./hr.). $Q_{\text{lactic acid}}$ denotes the rate of lactic acid formation as determined by chemical estimation, while $Q^{N_2}_{CO_2}$ applies to acid production calculated from changes in manometric pressure. The rate of change in the inorganic phosphate content of the reaction mixture is given by Q_P (μl. H_3PO_4/mg. dry wt./hr., 1 μg. atom $P = 22{\cdot}4\ \mu$l.). Q_{HDP} similarly denotes the change in its HDP content (1 μmol. HDP = 22·4 μl.). The dry weight of the suspension was determined by evaporation in the steam oven and was corrected for the salt content of the diluting fluid.

RESULTS

Anaerobic glycolysis and phosphorylation in mammary-gland suspensions

In earlier experiments suspensions of the mammary glands of rats and rabbits were prepared. While these preparations showed high glycolytic activity when incubated with reaction mixtures reinforced with yeast hexokinase (see Terner, 1952), their respiratory activity was poor. Under anaerobic conditions, in the presence of glucose, HDP, fluoride and pyruvate, a system employed by LePage (1948) in the study of tumour suspensions, a rapid esterification of inorganic phosphate accompanied the formation of lactic acid. When pyruvate was omitted from the fluoride-blocked system, vigorous carbon dioxide production and phosphorylation occurred without the formation of lactic acid (Table 1). The latter results point to the presence in the mammary gland of a very active enzyme system capable of producing phosphoglyceric acid and α-glycerophosphate by the dismutation of triose phosphate (see Green *et al.* 1937). Although the rate of carbon dioxide evolution due to the dismutation reaction of triose phosphate declined after an initial period of high activity, it was maintained for about 30 min. at a rate almost equal to that of the oxido-reduction between triose phosphate and pyruvate. Table 2 shows that during short periods of incubation fluoride did not inhibit the rate of carbon dioxide production from HDP. The addition of glucose and hexokinase as a phosphate-acceptor system approximately doubled the rate of acid formation whether in the absence or presence of fluoride. Insulin (free from glycogenolytic factor: 2·5 mg./100 ml.) was without effect.

Under aerobic conditions rat-mammary suspensions produced large amounts of lactic acid, and the rate of uptake of inorganic phosphate did not exceed the rate of anaerobic phosphorylation. 2:4-Dinitrophenol had no effect on lactic acid formation or phosphorylation (Table 1). Since considerable difficulty had been experienced during the preparation of these suspensions because of their tough connective tissue, it was concluded that the respiratory mechanism had been damaged. The mammary gland of the guinea pig, on the other hand, was found to be of softer texture and no difficulty was encountered in preparing actively respiring suspensions.

Table 1. *Phosphorylation and glycolysis in mammary suspensions of low respiratory activity*

(Rat-mammary suspension, dry wt. 19 mg. per vessel. Basal medium. All vessels also contained: glucose, 0·005 M; fructose 1:6-diphosphate (HDP), 0·0025 M; hexokinase, 18 units; ADP, 5×10^{-4} M; fluoride, 0·02 M; cytochrome *c*, 1×10^{-5} M. Additions: pyruvate, 0·005 M; fumarate, 0·005 M; 2:4-dinitrophenol (DNP), 2×10^{-4} M; iodoacetate, 0·002 M. Incubation period, 40 min.)

Vessel no.	Additions	Q_{O_2}	$Q^{O_2}_P$	$Q^{O_2}_{\text{lactic acid}}$
1	None	−1·8	−11·5	—
2	DNP	−3·1	−12·1	—
3	Pyruvate, fumarate	−2·2	−17·5	+24·8
4	Pyruvate, fumarate, DNP	−1·9	−17·9	+24·8
5	Iodoacetate	0	+2·7	0
		$Q^{N_2}_{CO_2}$	$Q^{N_2}_P$	$Q^{N_2}_{\text{lactic acid}}$
6	None	+20·5	−16·0	0
7	Pyruvate	+31·5	−22·0	+32·0
8	Iodoacetate	+3·5	+5·0	0

Table 2. *Anaerobic metabolism of fructose 1:6-diphosphate in mammary suspensions*

(Rat-mammary suspension, 31·7 mg. dry wt. per vessel. Basal medium; HDP (0·004 M) in all flasks. Additions: glucose, 0·02 M; fluoride, 0·01 M; hexokinase, 18 units; insulin (Novo), 2·5 mg./100 ml. Incubation period, 25 min.)

Vessel no.	Additions	$Q^{N_2}_{CO_2}$
1	None	24·2
2	Insulin	23·7
3	Fluoride	24·7
4	Fluoride, insulin	24·1
5	Glucose, hexokinase	54·4
6	Glucose, hexokinase, insulin	52·5
7	Fluoride, glucose, hexokinase	43·8
8	Fluoride, glucose, hexokinase, insulin	42·8

Aerobic phosphorylation and Pasteur effect. As shown in Tables 3–5, guinea pig mammary-gland suspensions are capable of vigorous aerobic phosphorylation in the absence of fluoride. Only small amounts of lactic acid were found to accumulate under aerobic conditions, as compared with the large amounts formed by anaerobic glycolysis; thus, mammary suspensions show a marked Pasteur effect. When the balance of respiration and glycolysis in the suspension was disturbed by the addition of an extract of rabbit-muscle powder, the respiration was unaffected and despite the greatly accelerated rates of aerobic and anaerobic lactic acid formation a Pasteur effect was found to persist (Table 3).

Effect of p-*nitrophenol.* The addition of *p*NP (1×10^{-4}–4×10^{-4} M) caused inhibition of aerobic phosphorylation and increased the accumulation of lactic acid, but was without effect in anaerobic experiments (Table 4). In the concentration range used, *p*NP did not inhibit and usually stimulated the respiration. The greatest stimulation of respiration, accompanied by effective inhibition of phosphorylation and increased lactic acid formation,

Table 3. *Aerobic and anaerobic glycolysis and phosphorylation in mammary suspensions*

(Guinea pig mammary suspension. Dry wt., 28·4 mg. per vessel. Basal medium; glucose, 0·01 M; HDP, 0·0025 M; hexokinase, 18 units. Rabbit-muscle acetone powder extract, 0·1 ml. Incubation period, 30 min. Initial inorganic P, 580 μg.)

Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	$Q^{N_2}_{CO_2}$	Q_P	$Q_{\text{lactic acid}}$
1	None	Aerobic	18·3	—	−26·2	2·8
2	Muscle extract	Aerobic	18·2	—	−24·7	16·1
3	None	Anaerobic	—	24·7	−3·5	24·7
4	Muscle extract	Anaerobic	—	49·0	−21·4	48·6
5	Iodoacetate, 0·001 M	Anaerobic	—	0	+13·1	1·5

Table 4. *Effect of inhibitors on phosphorylation and lactic acid accumulation in mammary suspensions*

(Guinea pig mammary suspension, dry wt. 44·8 mg. per vessel. Basal medium; glucose (0·01 M), HDP (0·0025 M) and hexokinase (18 units) in all flasks. Additions: pyruvate, 0·01 M; iodoacetate, 0·001 M; arsenite, 0·0025 M. Incubation period, 35 min. Initial inorganic P, 914 μg.)

Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	Q_P	$Q_{\text{lactic acid}}$
1	None	Aerobic	11·1	−12·6	2·1
2	*p*NP, 2×10^{-4} M	Aerobic	16·1	−5·9	7·9
3	*p*NP, 4×10^{-4} M	Aerobic	16·5	+6·1	10·5
4	Pyruvate	Aerobic	11·0	−12·6	4·5
5	Pyruvate; *p*NP, 2×10^{-4} M	Aerobic	11·9	+1·1	10·5
6	Pyruvate; *p*NP, 4×10^{-4} M	Aerobic	12·1	+7·8	10·7
7	Iodoacetate	Aerobic	5·6	−3·1	0·7
8	Pyruvate; iodoacetate	Aerobic	5·0	−2·3	1·6
9	Arsenite	Aerobic	7·9	+2·9	7·9
10	None	Anaerobic	—	−1·5	19·9
11	*p*NP, 4×10^{-4} M	Anaerobic	—	+1·9	20·4
12	Iodoacetate	Anaerobic	—	+5·1	1·6

Table 5. *Effect of pyruvate and fluoride on aerobic phosphorylation and lactic acid formation in mammary suspensions*

(Guinea pig mammary suspension, dry wt. 38 mg. per vessel. Basal medium; glucose (0·01 M), HDP (0·0025 M) and hexokinase (18 units) in all flasks. Additions: pyruvate, 0·01 M; fluoride, 0·01 M. Incubation period, 40 min. Initial inorganic P, 735 μg.)

Vessel no.	Additions	$-Q_{O_2}$	Q_P	$Q_{\text{lactic acid}}$
1	None	13·4	−15·2	5·2
2	*p*NP, 1×10^{-4} M	17·0	−11·4	7·8
3	*p*NP, 2×10^{-4} M	18·0	−5·4	10·9
4	*p*NP, 4×10^{-4} M	16·3	+4·8	11·9
5	Pyruvate	10·8	−11·9	8·5
6	Pyruvate; *p*NP, 1×10^{-4} M	12·4	−7·2	10·3
7	Pyruvate; *p*NP, 2×10^{-4} M	12·6	+0·3	11·0
8	Pyruvate; *p*NP, 4×10^{-4} M	11·8	+2·3	12·4
9	Pyruvate; fluoride	8·6	−17·7	6·9
10	Pyruvate; fluoride; *p*NP, 1×10^{-4} M	7·5	−12·0	8·1
11	Pyruvate; fluoride; *p*NP, 2×10^{-4} M	7·5	−8·1	9·1
12	Pyruvate; fluoride; *p*NP, 4×10^{-4} M	7·3	−7·4	9·0

Table 6. *Effect of fluoride on metabolism of mammary suspensions*

(Guinea pig mammary suspension, dry wt. 46·8 mg. per vessel. Basal medium; glucose (0·01 M), hexokinase 18 units, HDP (0·005 M), pyruvate (0·01 M) in all flasks. Additions: iodoacetate, 0·001 M; arsenite, 0·0025 M; *p*-nitrophenol (*p*NP), 4×10^{-4} M. Incubation period, 40 min. Initial inorganic P, 750 μg.)

Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	Q_P	$Q_{\text{lactic acid}}$
1	None	Aerobic	9·0	−5·8	7·5
2	*p*NP	Aerobic	10·0	+7·3	7·7
3	Fluoride, 0·005 M	Aerobic	7·2	−13·0	3·7
4	Fluoride, 0·005 M; *p*NP	Aerobic	7·0	−1·8	5·5
5	Fluoride, 0·01 M	Aerobic	6·7	−16·3	3·3
6	Fluoride, 0·01 M; *p*NP	Aerobic	6·7	−3·6	5·4
7	Fluoride, 0·02 M	Aerobic	5·6	−15·5	4·1
8	Fluoride, 0·02 M; *p*NP	Aerobic	5·8	−4·9	5·2
9	Iodoacetate	Aerobic	5·4	+3·8	2·0
10	Arsenite	Aerobic	4·7	+8·4	6·4
11	Arsenite; iodoacetate	Aerobic	2·4	+7·0	1·9
12	None	Anaerobic	—	+7·2	11·6
13	Fluoride, 0·005 M	Anaerobic	—	+1·7	11·6
14	Fluoride, 0·01 M	Anaerobic	—	−3·2	8·0
15	Fluoride, 0·02 M	Anaerobic	—	−2·5	7·2

Table 7. *Effect of fluoride on dephosphorylation and* P/O *ratios*

(Calculated from data of experiment shown in Table 6. Q_{-P} denotes the rate of dephosphorylation, calculated as the difference in the corresponding anaerobic vessels between the theoretical and observed Q_P; corrected Q_P = observed aerobic Q_P minus Q_{-P}.)

Vessel no.	Concn. of fluoride (M)	*p*NP	Q_{-P}	Corr. Q_P	$-Q_{O_2} \times 2$	P/O ratio
1	None	−	18·8	−24·6	18·0	1·4
2	None	+	18·8	−11·5	20·0	0·6
3	0·005	−	13·3	−26·3	14·2	1·8
4	0·005	+	13·3	−11·5	14·0	0·8
5	0·01	−	4·8	−21·1	13·4	1·5
6	0·01	+	4·8	−8·4	13·4	0·6
7	0·02	−	4·7	−20·2	11·2	1·8
8	0·02	+	4·7	−9·6	11·6	0·8

Table 8. *Effect of inhibitors on aerobic metabolism*

(Guinea pig mammary suspensions. Dry wt.: 43·5 mg. per vessel in Expt. 1; 44 mg. per vessel in Expt. 2. Vessels 1–5 of Expt. 1 and vessels 1 and 2 of Expt. 2 contained the basal medium without added substrates. All other vessels contained the complete reaction mixture which includes: glucose, 0·02 M; HDP, 0·0025 M; hexokinase, 18 units. Additions: *p*NP, 4×10^{-4} M; fluoride, 0·005 M; fluoroacetate, 0·01 M; malonate, 0·01 M; arsenite, 0·0025 M. Incubation period, 30 min. in Expt. 1; 40 min. in Expt. 2. Initial inorganic P, 750 μg.)

Expt. no.	Vessel no.	Reaction mixture	Additions	Experimental conditions	$-Q_{O_2}$	Q_P	$Q_{\text{lactic acid}}$
1	1	Basal	None	Aerobic	5·7	—	—
	2	Basal	Fluoride	Aerobic	5·0	—	—
	3	Basal	Fluoroacetate	Aerobic	3·2	—	—
	4	Basal	Malonate	Aerobic	2·2	—	—
	5	Basal	Arsenite	Aerobic	1·1	—	—
	6	Complete	None	Aerobic	10·4	−16·9	+6·7
	7	Complete	*p*NP	Aerobic	14·0	+2·2	+14·4
	8	Complete	Malonate	Aerobic	5·8	−2·5	+7·4
	9	Complete	Malonate, *p*NP	Aerobic	5·8	+1·5	+9·3
	10	Complete	None	Anaerobic	—	−5·4	+20·6
2	1	Basal	None	Aerobic	9·8	+4·7	−0·4
	2	Basal	*p*NP	Aerobic	8·1	+10·8	−0·1
	3	Complete	None	Aerobic	12·7	−13·9	+4·3
	4	Complete	*p*NP	Aerobic	18·0	+6·6	+12·4
	5	Complete	Malonate	Aerobic	6·0	+2·6	+3·3
	6	Complete	Malonate, *p*NP	Aerobic	5·0	+3·8	+3·4
	7	Complete	Fluoroacetate	Aerobic	8·7.	−4·5	+9·3
	8	Complete	Fluoroacetate, *p*NP	Aerobic	9·8	+6·5	+9·8
	9	Complete	None	Anaerobic	—	+3·1	+16·6

was produced by 2×10^{-4} M-*p*NP. Lower concentrations (1×10^{-4} M) only partly inhibited phosphorylation and the Pasteur effect (Table 5).

Effect of other inhibitors. Iodoacetate (0·001–0·002 M) which abolished glycolysis caused the liberation of large amounts of inorganic phosphate. Arsenite which is known to inhibit the oxidation of pyruvate (see Stocken & Thompson, 1949) has also been reported to be a powerful inhibitor of aerobic phosphorylation (Lehninger, 1949). In the present experimental conditions, arsenite (0·0025 M) inhibited phosphorylation and also increased the accumulation of lactic acid. Like iodoacetate, arsenite inhibited the respiration of the suspension, but left a considerable residual respiration which was reduced to a low level only by the combined action of the two inhibitors (Table 6). Fluoroacetate (0·01 M), an inhibitor of the Krebs cycle (see Peters, 1952), inhibited the Pasteur effect almost as effectively as *p*NP, but only partly inhibited the uptake of inorganic phosphate, the remaining phosphorylation being inhibited by *p*NP. Malonate (0·01 M), however, although it inhibited respiration and phosphorylation, did not increase lactic acid formation (Table 8).

Effect of inhibitors on the endogenous respiration. In the absence of added substrates the respiration of the suspensions in the basal medium was relatively high ($-Q_{O_2}$, 5–10). Fluoride and *p*NP had no effect on the endogenous respiration, while fluoroacetate, malonate and arsenite were strongly inhibitory. Suspensions respiring in the absence of added substrates and phosphate acceptors did not maintain the initial level of organic phosphate; its breakdown was further accelerated by *p*NP (Table 8).

Effect of pyruvate and fluoride. The addition of pyruvate to a glycolysing suspension did not increase the respiration nor the phosphorylation. The amount of lactic acid accumulating under aerobic conditions was, however, increased, often to the extent that *p*NP had no further effect. Pyruvate also suppressed the stimulation of respiration by *p*NP (Tables 4 and 5). Fluoride in the presence of pyruvate inhibited the respiration, higher concentrations (0·02 M) causing an inhibition of the same order of magnitude as arsenite or iodoacetate, while generally increasing the net uptake of inorganic phosphate (Table 6). The increased uptake of inorganic phosphate was often not very pronounced (Table 5), but in the presence of *p*NP or under anaerobic conditions fluoride appeared to retard the breakdown of organic phosphate.

In some guinea pig mammary suspensions fluoride in higher concentrations had an adverse effect on anaerobic glycolysis which partly offset its protective action against phosphatases with a resulting low net uptake of inorganic phosphate in relation to the amount of lactic acid produced (Table 6). In rat-mammary suspensions, however, the net uptake of inorganic phosphate in the presence of 0·02 M fluoride could be as high as 70–80% of the equivalent of the amount of acid produced both in the presence or absence of added pyruvate (Table 1).

Metabolism of fructose 1:6-*diphosphate.* Although in the experiments described above the addition of HDP was not essential for the demonstration of glycolysis and phosphorylation, the rates of glycolysis were somewhat higher in its presence than in its absence. When the phosphate-acceptor system was omitted and HDP was the only added substrate, the initial level of inorganic phosphate was approximately maintained in respiring suspensions and only small amounts of lactic acid were formed, while on addition of *p*NP or under anaerobic conditions the increased rate of lactic acid formation was accompanied by the liberation of inorganic phosphate. A marked stimulation of respiration by *p*NP could also be observed in the absence of the phosphate-acceptor system (Table 9).

Table 9. *Metabolism of fructose* 1:6-*diphosphate*

(Guinea pig mammary suspensions, dry wt. 46 mg. per vessel in Expt. 1; 42·8 mg. per vessel in Expt. 2. Basal medium; HDP, 0·005 M in Expt. 1; 0·0025 M in Expt. 2. Additions: *p*NP, 4×10^{-4} M; pyruvate, 0·01 M; fluoride, 0·005 M. Incubation period: 40 min. in Expt. 1; 30 min. in Expt. 2. Initial inorganic P: 900 μg. in Expt. 1; 760 μg. in Expt. 2.)

Expt. no.	Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	Q_P	$-Q_{HDP}$	$Q_{\text{lactic acid}}$
1	1	None	Aerobic	7·4	−1·3	3·9	0·8
	2	*p*NP	Aerobic	9·7	+6·3	4·6	4·0
	3	Pyruvate, fluoride	Aerobic	5·6	−4·3	3·4	2·9
	4	Pyruvate, fluoride, *p*NP	Aerobic	8·0	+3·4	4·9	5·4
	5	None	Anaerobic	—	+10·9	6·2	12·1
	6	Pyruvate, fluoride	Anaerobic	—	+3·7	6·1	11·0
2	1	None	Aerobic	7·8	−0·3	4·1	1·3
	2	*p*NP	Aerobic	14·0	+12·9	6·0	6·4
	3	Pyruvate	Aerobic	7·1	−0·5	3·9	4·2
	4	Pyruvate, *p*NP	Aerobic	14·5	+14·9	6·2	7·8
	5	None	Anaerobic	—	+8·9	6·3	11·3

When arsenate replaced the phosphate in the reaction mixture, the rate of aerobic glycolysis was increased, and the addition of *p*NP had no further effect. A Pasteur effect, however, remained since the rates of both HDP-breakdown and lactic acid accumulation did not reach anaerobic levels (Table 10). The effect of arsenate on aerobic glycolysis resembled the effect of *p*NP in the presence of phosphate in accelerating the rate of lactic acid formation more than the equivalent rate of HDP-breakdown so that the amount of substrate available for oxidation was decreased without a corresponding inhibition of respiration. Thus, two effects common to arsenate and *p*NP were to inhibit the Pasteur effect and to increase the ratio of oxygen consumed/substrate oxidized to a value which approached the theoretical ratio for the complete oxidation of substrate (see Terner, 1951*c*).

Pasteur effect and phosphorylation in brain suspensions. While the rate of anaerobic glycolysis in mammary-gland suspensions supplemented with yeast hexokinase was maintained at almost linear rates for at least 60 min., brain suspensions were less stable and showed a rapid decline of the initially high rate of glycolysis which could not be prevented by the addition of hexokinase (Terner, 1952). Since at the same time the rate of respiration decreased less rapidly, it was necessary, in order to compare aerobic and anaerobic metabolic rates, to limit the duration of incubation to a short period during which anaerobic glycolysis was maintained (30 min. or less). Table 11 shows that with these precautions it was possible to observe in brain suspensions essentially the same phenomena as in mammary-gland suspensions, namely a marked Pasteur effect sensitive to *p*NP and arsenite, stimulation of respiration by 2×10^{-4} M-*p*NP and its inhibition by fluoride. However, no appreciable net uptake of inorganic phosphate occurred in the absence of fluoride and even in its presence the uptake was relatively small. Nevertheless, phosphorylation occurred as shown by the liberation of large amounts of inorganic phosphate in the presence of inhibitors of oxidative phosphorylation. Thus, the results extend to mammary-gland and brain suspensions the view of Meyerhof & Fiala (1950), based on experiments with disintegrated yeast preparations, that the Pasteur effect and aerobic phosphorylation are closely connected.

When brain suspensions were incubated with HDP in the absence of added phosphate acceptors, anaerobic carbon dioxide production ceased after a short period of relatively high activity, while the respiration continued. In one experiment $-Q_{O_2}$ calculated for the period of 20 min. was: without HDP, 6·4; with HDP, 8·7; with HDP + *p*NP (2×10^{-4} M) 12·1. During the same interval $Q^{N_2}_{CO_2}$ was 18·0. In contrast to the stimulation of respiration

Table 10. *Effect of arsenate on metabolism of fructose* 1:6-*diphosphate*

(Guinea pig mammary suspension, dry wt., 46·7 mg. per vessel. Usual basal medium except that phosphate buffer was omitted. All vessels contained: HDP, 0·0045 M; Additions: phosphate, 0·0038 M; arsenate, 0·0038 M; *p*NP, 4×10^{-4} M. Incubation period, 50 min.)

Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	$-Q_{HDP}$	$Q_{lactic\ acid}$
1	Phosphate	Aerobic	8·8	3·8	1·0
2	Phosphate; *p*NP	Aerobic	12·7	6·4	8·4
3	Phosphate	Anaerobic	—	7·3	13·6
4	Arsenate	Aerobic	8·3	5·4	6·9
5	Arsenate; *p*NP	Aerobic	10·0	5·4	7·1
6	Arsenate	Anaerobic	—	6·7	13·4

Table 11. *Effect of inhibitors on phosphorylation and lactic acid accumulation in brain suspension*

(Guinea pig brain suspension, dry wt. 37·6 mg. per vessel. Basal medium; glucose (0·01 M), HDP (0·005 M) in all flasks; cytochrome *c* (1×10^{-5} M) in all aerobic flasks. Additions: pyruvate, 0·01 M; fluoride, 0·01 M; arsenite, 0·0025 M; iodoacetate, 0·001 M. Incubation period, 30 min. Initial inorganic P, 750 μg.)

Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	Q_P	$Q_{lactic\ acid}$
1	None	Aerobic	15·3	−2·1	5·5
2	*p*NP, 2×10^{-4} M	Aerobic	18·2	+16·1	10·6
3	*p*NP, 4×10^{-4} M	Aerobic	15·3	+20·0	11·9
4	Pyruvate, fluoride	Aerobic	8·2	−6·6	5·7
5	Pyruvate, fluoride, *p*NP, 2×10^{-4} M	Aerobic	12·8	+1·6	7·4
6	Pyruvate, fluoride, *p*NP, 4×10^{-4} M	Aerobic	11·3	+1·6	8·7
7	Arsenite	Aerobic	4·8	+23·9	10·1
8	Iodoacetate	Aerobic	5·7	+10·1	1·2
9	Arsenite, iodoacetate	Aerobic	3·1	+18·0	1·2
10	None	Anaerobic	—	+18·4	21·7
11	Pyruvate, fluoride	Anaerobic	—	−3·6	20·1
12	Iodoacetate	Anaerobic	—	+18·4	1·0

by *p*NP shown in this experiment, Tyler (1950) observed no augmentation, but only inhibition of respiration on adding 2:4-dinitrophenol to brain suspensions in the presence of HDP.

Phosphorus/oxygen ratios. Measurements of P/O ratios are usually made in highly purified systems in which phosphatase activity is low or suppressed by the addition of fluoride (see Krebs, Ruffo, Johnson, Eggleston & Hems, 1953). In the suspensions used in the present work, phosphatase action considerably reduced the uptake of inorganic phosphate, although under aerobic conditions, the rate of phosphorylation exceeded the rate of dephosphorylation. An estimate of the rate of destruction of organic phosphate was obtained from the differences between the amount of inorganic phosphate expected to be esterified during anaerobic glycolysis and the amount of inorganic phosphate actually taken up. Since the formation of 1 mole of lactic acid should result in the esterification of 1 mole of inorganic phosphate, the difference between the amount of phosphate equivalent to the amount of lactic acid formed and the amount of phosphate actually disappearing may be taken as a measure of the dephosphorylating capacity of the system. The quantity thus obtained, when added to the observed net uptake of phosphate, gives the amount of phosphate which would have been taken up if dephosphorylation could have been completely prevented. The P/O ratios calculated in this manner for mammary-gland and brain suspensions were of the order of 1·5. Lynen & Koenigsberger (1951), who determined the rate of dephosphorylation in respiring yeast cells by adding cyanide to inhibit respiration and glycolysis and measuring the ensuing liberation of inorganic phosphate, obtained P/O ratios of the order of 1·0. The corrected rates of aerobic phosphorylation for the experiment illustrated in Table 6 are given in Table 7. It can be seen that lower concentrations of fluoride (up to 0·005 M) afforded little protection while higher concentrations (0·01–0·02 M) caused about 75 % inhibition of dephosphorylation. As is to be expected, the P/O ratios, calculated as described above, were of the same order of magnitude in the presence and absence of fluoride.

DISCUSSION

Pasteur effect and aerobic phosphorylation. Until recently it has been found difficult to study oxidative phosphorylation in broken-cell preparations which also exhibit a Pasteur effect. Meyerhof & Fiala (1950) were the first to demonstrate both phenomena in the same preparation of disintegrated yeast cells. The phosphate uptake was small but distinct, and was inhibited by *p*NP. A disadvantage noted by the authors was that 'in such cases, where the yeast is nearly completely killed and the phosphate can be washed out, fermentation prevails too much over respiration and fermentative phosphorylation too much over oxidative phosphorylation'. In the present experiments oxidative phosphorylation and a Pasteur effect have been demonstrated in suspensions of mammary tissue which were practically free from intact cells. Rapid rates of oxidative phosphorylation could be observed, which greatly exceeded the rates of glycolytic phosphorylation. Mammary suspensions thus provide a system which is better balanced and more suitable for the study of the phenomena concerned. In these preparations the 'Pasteur mechanism' was so powerful that on addition of a muscle extract which doubled the rate of anaerobic glycolysis, the amount of lactic acid accumulating under aerobic conditions did not exceed one-third of the amount produced by anaerobic glycolysis.

Birmingham & Elliott (1951) described the suppression of lactic acid formation in brain suspensions glycolysing under aerobic conditions; no measurements of phosphorylation were made. Their preparations, on microscopic examination 'showed the presence of numbers of nerve and other cells', an observation also made with brain suspensions used in the present work. Although subjected to the same experimental conditions, brain suspensions differed from mammary suspensions by their inability to promote a rapid net esterification of inorganic phosphate. This no doubt resulted from an excessive rate of dephosphorylation due to the high adenosine triphosphatase (ATP-ase) content of the brain preparations, which was found by Meyerhof & Geliazkowa (1947) to be the cause of the rapid decline of anaerobic glycolysis. In mammary suspensions, however, the ATP-ase activity appeared to be sufficiently low to permit the net esterification of appreciable amounts of inorganic phosphate without the necessity of adding fluoride.

In the absence of fluoride, a full Pasteur effect was observed in both mammary and brain suspensions. In the presence of fluoride, which made it necessary also to add pyruvate, the aerobic lactic acid level was raised and was in most cases not further increased to an appreciable extent by *p*NP. It has been shown that this masking of the Pasteur effect was due to the presence of an excess of pyruvate. Since fluoride and pyruvate are as a rule added to cell-free preparations in order to check ATP-ase activity (see Potter & Siekevitz, 1952), it is not surprising that Clowes & Keltch (1951), in experiments with glycolysing suspensions, did not observe any effect on the aerobic formation of lactic acid by 4:6-dinitro-*o*-cresol added in concentrations which inhibited phosphorylation.

Metabolic rates under aerobic conditions and effect of nitrophenols. It has been assumed by various

workers that a rate-determining step of the glycolytic chain of reactions is retarded under aerobic conditions. Thus, in order to explain the decrease of the rate of glucose disapperance in yeast cells, Lynen & Koenigsberger (1951) suggested that the hexokinase reaction might be inhibited in the presence of air. However, in the present experiments the rate of HDP-breakdown was found to be influenced by the presence and absence of oxygen in the same way as the breakdown of glucose. Further, when the esterification of inorganic phosphate was measured in a system supplemented with glucose and yeast hexokinase, the hexokinase reaction did not appear to be impaired in the presence of air. Factors other than the hexokinase reaction must therefore be involved. Meyerhof & Fiala (1950) found that the activity of aldolase in fermenting yeast was not reduced by oxygen, and therefore thought it improbable that aldolase regulates the speed of fermentation.

According to Johnson (1941) and Lynen (1941) the rate of oxidation of triose phosphate is retarded by high ATP/ADP ratios and low levels of inorganic phosphate resulting from aerobic phosphorylation. Accordingly, it is now accepted that phosphate acceptors and phosphatases play an important part in the regulation of rates of substrate oxidation by controlling the availability of ADP and of inorganic phosphate (see Potter & Siekevitz, 1952). Recently, Siekevitz & Potter (1953) and Lardy & Wellman (1953) reported that the oxidation of various substrates can be accelerated by the addition of either phosphate acceptors or of 2:4-dinitrophenol; the latter was found to enhance the latent 'ATP-ase' activity of the system. This concept would explain satisfactorily the present observations that in mammary suspensions, metabolizing HDP in the absence of added phosphate acceptors, *p*NP accelerated the breakdown of HDP and at the same time stimulated the respiration and liberation of inorganic phosphate. However, while Lardy & Wellman (1952) found that in rat-liver mitochondria the accelerating effect of phosphate acceptors on the respiration could be duplicated by 2:4-dinitrophenol, the present experiments show that in whole suspensions incubated with substrates in the presence of phosphate acceptors the respiration can be considerably augmented by the further addition of a nitrophenol. This may be partly explained by the observations reported previously that the increased respiration resulting from the addition of nitrophenols to mammary-gland slices (Terner, 1951*c*), bull spermatozoa (Melrose & Terner, 1953) and mammary suspensions (Terner, 1953*b*), metabolizing various substrates, was not accompanied by a corresponding increase in the amount of substrate oxidized, with the result that the ratio of oxygen consumed/substrate oxidized was increased, and approached the theoretical ratio corresponding to the complete oxidation of the substrate. Apart from the accumulation of intermediates, the incomplete oxidation of a substrate may be due to its partial utilization in synthetic reactions which, if dependent on energy derived from oxidative phosphorylation, will be inhibited by nitrophenols. Synthetic reactions may also be expected to compete with the added phosphate-acceptor system and reduce the P/O ratio. The accepted average P/O ratio for the oxidation of pyruvate by the Krebs cycle is 3·0 (Ochoa & Stern, 1952). The P/O ratios which were calculated indirectly and may be assumed to represent the maximal value attainable under present experimental conditions were of the order of 1·5 (see p. 477).

The suggestion that the Pasteur effect is due to the retardation of the oxidation of triose phosphate owing to the lack of inorganic phosphate within the cell has been followed up by Lynen and his associates in experiments with intact yeast cells (Holzer & Lynen, 1950; Lynen & Koenigsberger, 1951; Holzer & Holzer, 1953). In view of the demonstration of a Pasteur effect sensitive to nitrophenols in broken-cell preparations of yeast and of mammary tissue, this may require further consideration. In broken-cell preparations, in contrast to suspensions of yeast cells, the glycolytic enzymes are for the most part in solution. In most of the present experiments the level of inorganic phosphate at the end of the incubation period was relatively high, and although a concentration gradient in respect of inorganic phosphate has been shown to exist between the mitochondria and the surrounding solution (Bartley & Davies, 1952), the glycolytic enzyme system was unlikely to suffer from a lack of inorganic phosphate even during vigorous aerobic phosphorylation. Moreover, when HDP was metabolized in the absence of an added phosphate acceptor, the initial level of inorganic phosphate was not diminished, but a full Pasteur effect could nevertheless be demonstrated. Thus it seems improbable that under the present experimental conditions the rate of breakdown of HDP was retarded by a lack of inorganic phosphate.

Arsenate is known to accelerate glycolysis by making it independent of phosphorylation (Warburg & Christian, 1939*a*, *b*; Meyerhof & Junowicz-Kocholaty, 1942) and the inhibition of the Pasteur effect in its presence may therefore be expected. The observation in mammary suspensions that nitrophenols and arsenate accelerate the accumulation of lactic acid more than the breakdown of HDP remains unexplained. Similar observations have been reported previously, when it was found that 2:4-dinitrophenol could cause increased aerobic lactic acid formation in mammary slices while at the same time inhibiting the disappearance of glucose,

which was the only added substrate (Terner, 1952). It seems possible that the oxidation-reduction step of glycolysis also plays a part and this appears to be supported by the observation that added pyruvate increased the amount of lactic acid formed under aerobic conditions. Since only part of the added pyruvate was reduced to lactate, the rate of oxidation of triose phosphate may be a limiting factor. According to older views concerning the Pasteur effect, the aerobic reoxidation of reduced cozymase, formed during the oxidation of glyceraldehyde phosphate, may prevent the reduction of pyruvate to lactic acid (see Ball, 1939). To explain the inhibition of the Pasteur reaction by 2:4-dinitrophenol the suggestion was made in preliminary notes (Terner, 1951*a*, 1951*b*) that the nitrophenol might interfere with the aerobic reoxidation of reduced cozymase, thus favouring its reoxidation by the coupled reduction of pyruvate to lactate. However, attempts in unpublished experiments to obtain evidence of an inhibition by nitrophenols of the aerobic oxidation of reduced cozymase have been unsuccessful and this hypothesis now appears to be unlikely.

SUMMARY

1. A Pasteur effect coupled with aerobic phosphorylation has been demonstrated in cell-free suspensions of mammary gland incubated with fructose 1:6-diphosphate (HDP) in the presence of glucose and yeast hexokinase as the phosphate-acceptor system.

2. Both phenomena were inhibited by *p*-nitrophenol (*p*NP, $2–4 \times 10^{-4}$ M) which at the same time could cause up to 50% stimulation of respiration; *p*NP had no effect on the respiration of the suspensions in the absence of added substrates.

3. Similar results were obtained with brain suspensions, which, however, contained considerable numbers of unbroken cells.

4. A Pasteur effect was also demonstrated in mammary suspensions incubated with HDP, in the absence of added phosphate acceptors. In these experiments the initial level of inorganic phosphate was approximately maintained under aerobic conditions; the addition of *p*NP resulted in stimulation of respiration and an increase in the amount of inorganic phosphate in the medium.

5. In the absence of added phosphate acceptors, *p*NP and also arsenate accelerated the aerobic breakdown of HDP; the increase in the amount of lactic acid accumulating exceeded the increase expected from the acceleration of the breakdown of HDP. No effect of *p*NP was observed under anaerobic conditions.

6. The addition of pyruvate, or pyruvate plus fluoride (0·005–0·02 M), resulted in increased aerobic formation of lactic acid; in their presence, *p*NP produced little or no further inhibition of the Pasteur reaction and its stimulating effect on respiration was suppressed.

7. Phosphorus/oxygen ratios for the overall reaction were determined by an indirect method and were found to be of the order of 1·5 under conditions supporting aerobic phosphorylation.

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Sedimentation and Diffusion of Human Albumins

4. LOW CONCENTRATION BEHAVIOUR OF SERUM ALBUMINS IN CASES OF LIVER DISEASE

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It has long been realized that the liver, even if not directly responsible for the synthesis of serum albumin, is at least intimately involved in its metabolism. The possible role of the liver in albumin formation was discussed by Madden & Whipple (1940), who concluded that it is highly likely that the liver constitutes a site of production. Further evidence in favour of this view is forthcoming from the work of Roberts & White (1949) and the radioactive studies by Peters & Anfinsen (1950).

The very close connexion between the liver and albumin formation suggests the possibility of alterations in the protein when that organ is diseased. Quantitative changes in the serum proteins have been reported by a number of authors who used the electrophoretic technique. Thus cases of cirrhosis have been studied by Gray & Barron (1943), Thorn, Armstrong & Davenport (1946), Martin (1949*a*), Rafsky, Weingarten, Krieger, Stern & Newman (1950), and Franklin *et al.* (1951). Sera from patients with infective hepatitis formed the basis of work by Gray & Barron (1943), Martin (1946), Cohen & Thompson (1947), Rafsky *et al.* (1950) & Franklin *et al.* (1951). A common feature of the patterns is the reduction in the relative concentration of albumin, although this decrease is by no means specific to diseases of the liver (Gutman, 1948).

Qualitative alterations in the serum albumin are indicated by two types of evidence. First, Luetscher (1939) and Hoch-Ligeti & Hoch (1948) found alterations in the electrophoretic characteristics of the albumin peaks when examined under special conditions. Secondly, albumin separated by electrophoresis of sera from patients with liver disease was shown to behave abnormally in its ability to inhibit the cephalin cholesterol flocculation of γ-globulin, and in its effect on the thymol flocculation test, etc. (Kabat, Hanger, Moore & Landow, 1943; Moore, Pierson, Hanger & Moore, 1945; Hanger, 1947; Martin, 1949*b*). Unfortunately, the conditions used to isolate the albumin were not designed to remove all the α-globulin, so that the conclusions which can be drawn are not quite so definite as might be desired.

The abnormal molecular weights found by sedimentation and diffusion measurements for serum and urinary albumins in nephrosis (Charlwood 1952*b*), and the above considerations, have prompted a similar investigation in cases of liver disease, with the object of obtaining evidence of any abnormalities in albumin synthesis.

The main complication likely to be caused by the conditions prevailing in liver diseases lay in the generally raised bilirubin content of the sera. This could affect the actual molecular weight of the albumin, to which bilirubin is largely bound (Martin, 1949*c*). The colour, too, might affect optical conditions, more especially in the diffusiometer, and give rise to anomalous results. Both these points are discussed later.

EXPERIMENTAL

Preparation of materials. Blood was collected from patients selected on the basis of a preliminary diagnosis of primary liver disease. It was processed as previously described (Charlwood, 1952*a*), the buffer in all cases being veronal (pH 8·6, $I = 0{\cdot}1$). Separations were controlled by the photoelectric arrangement described by Warren & Charlwood (1953), as in other similar work (Charlwood, 1954). Samples were diluted and redialysed, prior to sedimentation and diffusion, against a solution containing 0·05 *I*-NaCl + 0·02 *I* phosphate buffer, pH 8·0.

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To examine the possible effects of bilirubin, two solutions were made up, according to the technique of Martin (1949*b*), to give albumin concentrations of about 0·2 and 0·6%, respectively, and to contain sufficient bilirubin for maximum binding. To a weighed amount of bilirubin dissolved in cold 0·01 N-NaOH was added 20 times its weight of ascorbic acid dissolved in phosphate buffer, followed by the protein in the same buffer. The solution was diluted to the requisite volume for dialysis. The albumin was the Harvard preparation A used in earlier work (Charlwood, 1954). The bilirubin to which it was added was approximately double the maximum amount bound under these conditions, as given by the results of Martin (1949*b*). After dialysis for 3 days, the precipitate, containing excess of bilirubin, was removed by centrifugation and the deeply coloured solution dialysed for a further 2 days against fresh buffer. In the lower-concentration experiment the buffer used was the same as that in which the sedimentation and diffusion of the pathological albumins took place (see above). At the higher protein concentration the buffer mixture was double strength, i.e. 0·1 *I*-NaCl + 0·04 *I* sodium phosphate buffer, pH 8·0.

Sedimentation and diffusion experiments. These were made in accordance with previous practice (Charlwood, 1952*a*, *b*). As usual, a single ultracentrifuge run and two pairs of diffusion runs were carried out on each solution, except that it was felt that a single pair of diffusion runs would suffice for the albumin/bilirubin mixture at 0·6% concentration.

The work of Waugh & Yphantis (1952) and Shulman (1953) on rotor temperatures in the Spinco Ultracentrifuge have been briefly discussed by Charlwood (1954). It was again decided to record values of $S_{20,w}$ based on the mean of initial and final rotor temperatures, for reasons given in that discussion.

RESULTS

All sedimentation constants, corrected to water at 20°, are shown in Svedberg units, whilst diffusion constants, similarly corrected, are in units of 10^{-7} cm.^{2} sec.$^{-1}$. Concentrations (v) refer to wavelengths of retardation in the diffusiometer, a protein concentration of 1% corresponding to v ~ 38.

Diffusion

The results obtained with the albumin/bilirubin mixtures are shown in Table 1. Comparison with the figures given for the same albumin, free from added bilirubin (Charlwood, 1954), shows agreement within experimental error.

The figures shown in Table 2 for the pathological albumins refer only to those cases in which the preliminary diagnosis was subsequently confirmed. The mean value for the diffusion constants is 6·31, which is virtually identical with the mean value (6·32) found for normal human albumins at similar low concentrations (Charlwood, 1952*a*). The mean values for the cases of cirrhosis (6·30) and infective hepatitis (6·33) are also not significantly different from normal nor from each other. The single instance of carcinoma is well within the normal range. Indeed, the only individual value lying outside this range is the first, which is just below the lower limit of 6·11 (Charlwood, 1952*b*).

Table 1. *Sedimentation and diffusion of albumin-bilirubin complex*

(v = Concentrations of protein referred to wavelengths of retardation in the diffusiometer at 1% protein conc. v ~ 38.)

Buffer	v	$S_{20,w}$	$D_{20,w}$
0·05 *I*-NaCl + 0·02 *I* sodium phosphate, pH 8·0	8·46	4·25	6·13
0·1 *I*-NaCl + 0·04 *I* sodium phosphate, pH 8·0	21·82	4·05	6·02

Table 2. *Sedimentation and diffusion of serum albumins in various liver diseases*

(Phosphate buffer, pH 8·0, *I* = 0·02, and NaCl, *I* = 0·05.)

Case	Diagnosis	v	$S_{20,w}$	$D_{20,w}$
1	Cirrhosis	8·69	4·21	6·09
2	Cirrhosis	8·72	4·26	6·47
3	Cirrhosis	8·36	4·23	6·14
4	Infective hepatitis	9·27	4·18	6·31
5	Infective hepatitis	7·99	4·26	6·29
6	Infective hepatitis	7·75	4·27	6·26
7	Carcinoma	8·46	4·18†	6·24
8*	Cirrhosis	6·58	4·23	6·51
9	Infective hepatitis	9·27	4·16	6·42
10	Cirrhosis	8·04	4·19	6·26
11	Infective hepatitis	8·23	4·17	6·35
12	Infective hepatitis	8·59	4·12	6·24
13	Cirrhosis	7·52	4·10	6·30
14	Infective hepatitis	7·64	4·10	6·46

* Patient had been treated with bromsulphalein.
† Mean of two determinations.

Sedimentation

The results for the albumin-bilirubin complex, shown in Table 1, are again indistinguishable from those for the same albumin alone at corresponding concentrations (Charlwood, 1954).

In Table 2 are the results given by the pathological albumins in the chronological order in which they were obtained. There is a small, but significant difference ($0·01 > P > 0·001$) between their mean (4·19) and the mean (4·25) for normal albumins isolated under the same conditions (Charlwood, 1952*a*). For reasons discussed below, only the first six sedimentation constants in Table 2 are considered reliable to the usual degree of accuracy. These show no appreciable differences between the albumins from patients with infective hepatitis and those with cirrhosis, nor between either of these groups and normal. The last eight values in Table 2 (mean 4·16), taken together, are most definitely below normal ($P < 0·001$).

DISCUSSION

Before this work was begun, the possibilities of interference by bilirubin were considered. If there were much absorption of light by the solution in the

diffusiometer cell, the theory of the instrument would no longer be strictly applicable (Cecil & Ogston, 1951). However, examination of the light absorption curve of bilirubin in presence of human albumin (Martin, 1949*b*), and under buffer conditions similar to those it was proposed to use, showed relatively little absorption at the wavelength generally used in the diffusiometer ($\lambda = 5461$ Å). This high transparency was confirmed both visually, and by the density of the photographic record. Moreover, the degree of pigmentation of the serum albumins did not, except in a few instances, approach the maximum indicated by Martin (1949*b*). This could be seen by visual comparison of the colour intensity of the pathological albumin solutions with the albumin/bilirubin mixture made up at the lower concentration.

The other, more direct effect produced by bound bilirubin would be an increase in the molecular weight of the albumin. A simplified calculation was made to gain an idea of the possible magnitude of such an alteration. If the molecular weight of electrophoretically isolated human albumin be taken as 61500 (Charlwood, 1952*a*), and it be assumed that three molecules of bilirubin are bound to each one of albumin (Martin, 1949*b*), the molecular weight of the complex is about 62070. If the combination occurs without drastic change of molecular shape (i.e. the frictional ratio is unaltered), the diffusion constant of the complex should be about 0·3 % lower, and the sedimentation constant about 0·6 % higher than that of the original albumin. (This argument, of course, ignores the small amount of bilirubin originally attached to the normal albumin, the effect of which would be to decrease slightly the changes calculated.) Differences of this magnitude would not be detectable through small numbers of observations at the present degrees of accuracy (Table 1). Indeed, even on the whole set of measurements shown in Table 2, it would be necessary to show a difference from the normal average for $D_{20,w}$ of 0·9 % to register a value of $P < 0{\cdot}05$. The actual difference of about 0·16 % can, therefore, be attributed purely to experimental error. The fact that it is in the right direction, and of the order to be expected from incomplete binding of bilirubin, is purely a coincidence.

The pH of 8·0 was chosen for this work as it came within the scope of the observations of Martin (1949*b*) on the interaction of bilirubin and human-serum albumin. Comparison of measurements made under these conditions with those made on normal albumins in unbuffered sodium chloride solution (Charlwood, 1952*a*) is fully justified by the experiments of Charlwood (1954). Values obtained in different solutions (particularly in unbuffered sodium chloride, and in sodium chloride solution containing phosphate buffer, pH 8·0) gave virtually the same figure on extrapolation to zero concentration, and also at the low concentrations used in this work.

It has already been pointed out that the lower average value of the sedimentation constants in Table 2 is due to the preponderance of low figures in the group formed by the last eight. There is no correlation between these low figures and the relative numbers of cases of cirrhosis or infective hepatitis in this group and the first group of six. However, it was noticed that the drop in $S_{20,w}$ coincided fairly closely with the installation of a new drive-unit in the ultracentrifuge. This alteration was made between measurements on cases 6 and 7. Careful comparison of plates taken before and after the change showed that the quality of definition of the edge of the index mark was poorer and the apparent width of the meniscus had increased. The focus of each lens was checked, confirming that these effects were not due to maladjusment of the optical system. Other possible sources of error were also examined (Charlwood, 1952*a*). The conclusion was then reached that there must be sufficient vibration set up by the new drive-unit to account for the lowered values and other phenomena. A statistical calculation carried out on the last eight sedimentation constants in Table 2 gave value of P slightly greater than 0·05. Thus the evidence does not imply any progressive deterioration in the behaviour of the machine.

Since the above sedimentation constants refer to pathological materials, it was felt desirable to strengthen the opinion about the decreased reliability by means of runs on more normal proteins. To this end, β-lactoglobulin and bovine plasma albumin were used. The β-lactoglobulin solution (1·2 % protein in a mixture of 0·1 N sodium chloride, 0·1 N sodium acetate, 0·04 N acetic acid) had previously given a value of 2·78 in the Spinco machine (Charlwood, 1952*a*). On this occasion 2·73 was recorded. The difference, although not significant taken alone, is in the direction and of the size to be expected from Table 2. Bovine plasma albumin has been examined by several authors in the Spinco ultracentrifuge. Values of $S^0_{20,w}$, all lying between 4·27 and 4·32, have been obtained by Kegeles & Gutter (1951), Creeth (1952), Miller & Golder (1952), Taylor (1952) and Shulman (1953). From the figures given by these various authors it appears that $S_{20,w}$ for bovine plasma albumin at 0·46 % is about 4·17. A run in phosphate buffer (pH 7·0, $I = 0{\cdot}1$) at this concentration gave a value of 3·96. The difference of 5 % in $S_{20,w}$ is again consistent with a general lowering of 2·5 % superimposed on the random errors. The conclusion is, therefore, that the last eight sedimentation constants in Table 2 are probably about 2·5 % lower than they would otherwise be, merely on account of a slight defect in

the machine, and not because of the pathological nature of the albumins.

It has been shown that no measurable difference is detectable between the sedimentation and diffusion constants of serum albumins from normal persons and those suffering from the liver diseases represented in this survey. If it is assumed that measurements at a concentration of about 0·2% protein can be used here for calculations of the molecular weight (Charlwood, 1952*a*, *b*; 1954), and that the partial specific volumes of normal and pathological albumins are the same, it follows that the molecular weights are also indistinguishable. Now, Luetscher (1939) found that, in cases of cirrhosis of the liver, the ratio of the areas of the two peaks which separate during electrophoresis of human-serum albumin at pH 4·0 in 0·2 *I* acetate buffer differs from normal. However, since these components have essentially the same sedimentation and diffusion constants (Charlwood, 1954), there is no reason, on the basis of Luetscher's discovery, to anticipate abnormal molecular weights for serum albumins in cirrhosis of the liver. The data presented in this paper give no indication of a significant qualitative difference between albumin separated from normal serum and that from persons suffering from liver disease.

SUMMARY

1. The albumin fraction has been isolated electrophoretically from the sera of patients with cirrhosis of the liver, infective hepatitis, and one case of carcinoma of the liver.

2. Sedimentation measurements in the Spinco Ultracentrifuge, and also diffusion measurements in the Gouy diffusiometer, have been carried out on these albumins at a protein concentration of the order of 0·2%.

3. Possible interference of bilirubin with the determinations has been studied.

4. There is evidence that the albumins extracted from the sera of patients with liver diseases are normal as regards molecular weight, and the data presented give no indication of qualitative differences between the albumins isolated.

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The Mechanism of the Formation of Organic Acids by Mould Fungi

6. THE INFLUENCE OF CYANIDE ON *ASPERGILLUS NIGER*

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In continuation of previous studies of acid formation in *Aspergillus niger* (Walker, 1949; Chughtai, Pearce & Walker, 1950), the effects of cyanide on the utilization of glucose in cultures of this mould have been subjected to quantitative examination.

EXPERIMENTAL

The strain (N1) of *A. niger* used in our previous work was again employed and was grown at 30° on a sterilized medium made up with distilled water and containing (% w/v) NH_4NO_3, 0·2; KH_2PO_4, 0·1; $MgSO_4.7H_2O$, 0·025; and glucose, 10. The pH was adjusted to 4·4 with HCl. This medium was dispensed in quantities of 140 ml. in conical flasks of 600 ml. capacity and having necks 35 mm. in diameter. Felts were grown from spores and when, after about 4 days, strong mycelial mats had been formed, the culture medium was withdrawn and replaced aseptically by an equal volume of the salt solution without glucose. The flasks were then left overnight in the incubator in order to allow time for assimilation of absorbed sugar.

After 12 hr. the salt solution was withdrawn and replaced by an equal volume of glucose solution (10%, w/v) without nutrient salts, but containing NaCN at concentrations of 0·006, 0·010, 0·020 or 0·040 M. Since the felts which had been standing over the inorganic-salt solution retained some of the latter in their under-surface convolutions, this diluted the 10% solution of glucose which was used as replacement. A correction for the dilution was therefore applied in all cases. Normally, five flasks were employed for each concentration of cyanide, and controls without cyanide were also set up. The initial pH of the contents of the cyanide-containing flasks was approximately 9·5, but when incubation was resumed the activity of the mould speedily lowered it into the acid range. Samples were withdrawn at intervals for estimation of glucose utilized and acids formed. In other cases the replacement media consisted, respectively, of a solution of glucose, together with $NaNO_3$, and of a solution of citric acid. The analyses were performed by the methods indicated by Chughtai & Walker (1951).

RESULTS

The range of concentration of cyanide was adopted after preliminary tests had shown that development of the mould from spores was suppressed by cyanide at 0·015 M. However, Table 1 shows that formation of acid by 'ready-formed' mycelial felts was stimulated by cyanide at all the concentrations which were adopted, the optimum effect being observed at a concentration of 0·020 M. Formation of felt also was stimulated by cyanide (Table 2). According to

Table 1. *Consumption of glucose and formation of citric, oxalic and gluconic acids by* Aspergillus niger *in presence and in absence of sodium cyanide*

(Glucose present initially at a concn. of 8·87 g./100 ml., equivalent to 3·55 g. carbon/100 ml.)

Cyanide concn. (M)	Time of incubation (days)	Glucose utilized (g. carbon/100 ml.)	Acid formation: Citric (g. carbon/100 ml.)	Citric (% yield)*	Oxalic (g. carbon/100 ml.)	Oxalic (% yield)	Gluconic (g. carbon/100 ml.)	Gluconic (% yield)
0	6	0·812	0·174	21·5	0·047	5·8	0·028	3·6
	8	1·356	0·229	16·9	0·048	3·5	0·072	5·6
	11	1·521	0·265	17·4	0·053	3·5	0·188	12·3
0·010	6	1·428	0·337	23·6	0·097	6·8	0·433	30·3
	8	1·935	0·449	23·2	0·092	4·8	0·622	32·0
	11	2·381	0·378	15·9	0·096	4·0	0·577	24·2
0·020	6	2·037	0·469	23·0	0·099	4·9	0·536	26·2
	8	2·537	0·489	19·3	0·119	4·7	0·758	29·9
	11	3·317	0·394	11·9	0·123	3·7	0·548	16·5
0·040	6	1·902	0·338	17·8	0·084	4·4	0·570	30·0
	8	2·493	0·468	18·8	0·113	4·5	0·744	29·8
	11	3·295	0·378	11·5	0·113	3·2.	0·614	18·6

* Percentage yield based on carbon content of glucose utilized.

Table 2. *Influence of sodium cyanide on the weight of mycelium developed by* Aspergillus niger *in a glucose medium*

(Glucose present initially at a concn. of 8·85 g./100 ml., equivalent to 3·54 g. carbon/100 ml. Carbon content of felt at 3 days, before introduction of cyanide = 0·657 ± 0·112 g./flask.)

Cyanide concn. (M)	Glucose utilized (g. carbon/157 ml.)	Carbon in felt at 8 days (g./157 ml.)	Gain in carbon in felt in last 5 days		Sum of citric, oxalic and gluconic acids plus gain in felt (g. carbon/157 ml.)
			(g./157 ml.)	(% yield)*	
0	4·600	1·313	0·656	14·3	2·218
0·006	4·458	1·387	0·730	16·4	2·429
0·020	5·154	1·731	1·074	20·1	3·292

* Percentage yield on carbon content of glucose utilized.

Table 3. *Comparison of the respective effects of sodium cyanide and sodium nitrate on glucose consumption and acid formation by* Aspergillus niger

(Glucose present initially at a concn. of 8·68 g./100 ml., equivalent to 3·47 g. carbon/100 ml.)

Concn. of cyanide (M)	Concn. of nitrate (M)	Time of incubation (days)	Glucose utilized (g. carbon/100 ml.)	Gain in carbon in felt in 7 days (g./100 ml.)	Acid formation					
					Citric		Oxalic		Gluconic	
					(g. carbon/100 ml.)	(% yield)*	(g. carbon/100 ml.)	(% yield)	(g. carbon/100 ml.)	(% yield)
0	0	3	1·567	—	0·528	33·9	0·041	2·6	0·071	4·5
		5	1·743	—	0·643	36·9	0·056	3·2	0·181	10·4
		7	1·911	0·433	0·745	39·0	0·065	3·4	0·314	16·4
		10	2·348	—	0·951	40·5	0·072	3·1	0·274	11·6
		12	2·768	—	1·093	39·5	0·099	3·6	0·213	7·6
0·020	0	3	2·062	—	0·944	45·8	0·092	4·5	0·301	14·6
		5	2·700	—	1·172	43·4	0·125	4·6	0·607	22·4
		7	3·255	1·493	1·355	41·6	0·161	4·9	0·485	14·9
		10	3·359	—	1·331	39·6	0·106	3·2	0·514	15·3
		12	3·409	—	1·105	32·4	0·118	3·5	0·253	7·4
0	0·020	3	1·709	—	0·642	37·5	0·068	4·0	0·213	12·4
		5	2·348	—	0·804	34·2	0·116	4·9	0·215	9·2
		7	2·852	1·232	0·916	32·2	0·156	5·5	0·303	10·5
		10	3·174	—	1·066	33·6	0·144	4·5	0·241	7·6
		12	3·241	—	0·892	27·5	0·122	3·8	0·263	8·1

* Percentage yield based on carbon content of glucose utilized.

Table 4. *Consumption of citric acid by* Aspergillus niger *in presence and in absence of sodium cyanide*

(Citric acid present initially at a concn. of 2·708 g. of anhydrous acid/100 ml., equivalent to 1·015 g. carbon/100 ml.)

Cyanide concn. (M)	Time of incubation (days)	Citric acid utilized		Oxalic acid formed	
		(g. carbon/100 ml.)	(% of initial quantity)	(g. carbon/100 ml.)	(As % of citric acid consumed)
0	3	0·676	66·9	—*	—
	5	0·888	87·9	—	—
	14	1·015	100·0	—	—
0·020	3	0·044	4·4	—	—
	5	0·055	5·4	—	—
	14	0·632	62·6	0·062	9·8

* Denotes failure to detect oxalic acid by the diphenylamine test (Vogel, 1947) which is sensitive to 5 μg. and has a concentration limit of 1:10000.

Ivanov & Osnizkaja (1934) and to Ivanov, Ivanova & Larinova (1936), *A. niger*, when in conditions of nitrogen starvation, can utilize the nitrogen of cyanide. Hence, it seemed possible that nitrogen from this source might have contributed to the increase in weight of mycelial felt so the effects of equivalent amounts of sodium cyanide and sodium nitrate were compared (Table 3).

Finally, the utilization of citric acid by *A. niger* in presence of cyanide was studied (Table 4) since it was desired to ascertain whether the higher yields of citrate which were obtained in the media containing cyanide could be ascribed wholly or in part to suppression by this compound of the mould's ability to metabolize citric acid, for aconitase is inhibited by hydrogen cyanide at relatively low concentration (Krebs & Eggleston, 1944). The utilization of free citric acid by *A. niger* in cultures in which this acid was the sole source of energy was greatly restricted in presence of cyanide. During incubation of these cultures the presence of free hydrocyanic acid could be detected by its smell (the flasks were plugged with cotton wool) when the door of the incubator was opened. In the experiments with cyanide in a glucose solution no smell of free hydrocyanic acid was ever noted, although the activity of the mould caused the pH of these solutions (initially 9·5) to fall rapidly to values usually below 3.

The highest intensity of metabolic activity as evidenced by sugar consumption, by formation of mycelial felt and by production of acid, was attained by preformed felts of the mould in presence of cyanide at concentration 0·020 M. At this level (Table 1) sugar consumption at 6, 8 and 11 days was approximately double that in the control flasks, while at the same stages the yields of citric acid were respectively 2·7, 2·1 and 1·5 times those found in the controls. At these same stages the yields of oxalic acid were 2, 2·5 and 2·3 times those produced in absence of cyanide. Similarly, formation of gluconic acid was greatly enhanced by the effect of the cyanide, the amount estimated at 11 days being 2·9 times the quantity detected in the cyanide-free cultures. Moreover, the weights of felt synthesized and the total weights of acids formed in presence of cyanide are greater, calculated as percentages on the sugar consumed, than the corresponding yields of felt and acids afforded by the control flasks (Table 2), an indication that one of the effects of cyanide is to spare loss of carbon dioxide from the cultures.

DISCUSSION

The fact that addition of extra assimilable nitrogen, in the form of sodium nitrate to preformed felts of *A. niger* on a glucose solution (Table 3), served to diminish somewhat the percentage yield of citric acid renders it improbable that the stimulation of citrate formation by an equivalent quantity of sodium cyanide was due to the nutrient value of the nitrogen of the cyano group. The effects of cyanide, as manifested in considerable increases in sugar consumed, felt produced and gluconic, citric and oxalic acids formed, indicate general stimulation of physiological function in the mould rather than activation of any one specific process. Similar effects produced by cyanide were observed by Dammann, Rotini & Nord (1938), who reported that cyanide stimulated considerably the metabolic activity of *Fusarium graminearum* Schwabe, causing an increase in the weight of felt and at the same time a higher level of activity in the fermentation of pentoses, notably of L-arabinose, whereby also the yield of alcohol was markedly increased.

Sodium arsenite, an inhibitor of oxidative decarboxylation (Krebs, 1933*a*, *b*; Krebs & Johnson 1937), when used at a certain concentration in glucose cultures of *A. niger* (Chughtai & Walker, 1951) produces results similar in every respect to those now noted in presence of sodium cyanide. The occurrence of pyruvic acid in glucose cultures of *A. niger* after treatment with arsenite was reported by Chughtai *et al.* (1950) and more recently α-oxoglutaric acid was detected in similarly treated glucose cultures of this mould (Walker, Hall & Hopton, 1951). These facts would seem to indicate that one of the activities of cyanide when introduced into glucose cultures of *A. niger* is to exert a stabilizing influence on the α-keto acids of the tricarboxylic acid cycle. Cyanhydrin formation no doubt occurs, since Gale & Stephenson (1939) found cyanide (0·060 M) to be the only effective agent for stabilizing the oxaloacetic acid produced by the action of malic dehydrogenase.

Since the data given in Table 4 shows that hydrocyanic acid interferes powerfully with the action of those enzymes by which *A. niger* degrades citric acid, it might be expected to exercise a similar sparing action on the citric acid produced in glucose cultures of the mould. The fact that citric acid does not accumulate extensively in such cultures after the introduction of cyanide is due most probably to the speedy removal of the latter by combination with ketonic metabolites. Such a removal of hydrocyanic acid would also account for the fact that no smell of this acid has been detected coming from glucose cultures after addition of sodium cyanide, although such cultures rapidly produce sufficient acid to bring the pH to about 3. Summing up, it would seem reasonable to conclude that in glucose cultures prepared with preformed felts of *A. niger*, sodium cyanide promotes the tricarboxylic acid cycle by restricting more highly oxidative side-reactions.

SUMMARY

1. Preformed mycelial felts of *A. niger* treated with cyanide at four concentrations between 0·006 and 0·040 M, utilized more sugar, synthesized more mycelial substance and formed a greater total of acids (citric, oxalic and gluconic) in a given period than did controls. The most effective concentration used was 0·020 M.

2. In presence of 0·010 or 0·020 M cyanide the yields of all products were not only actually greater than with the controls but were also greater when expressed as percentages on the weight of sugar consumed.

3. The breakdown of citric acid by *A. niger* is impeded by cyanide.

4. Possible modes of action of the cyanide are discussed.

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Heat Transformation Products of Cottonseed Oil

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The high incidence of cancer of the stomach has indicated the possibility that heated foodstuffs contain a carcinogen. However, in this laboratory examination of fats heated to relatively high temperatures in cooking has not revealed the presence of agents of the aromatic hydrocarbon type, which show characteristic fluorescence. Biological tests of the carcinogenicity of heated cottonseed-oil products by feeding experiments on mice have been described by Peacock, Beck & Chalmers (1953) and additional tests made by subcutaneous injection into mice will be described later in this communication.

In the feeding experiments to which reference has been made, the following cottonseed-oil products have been used: (1) Cottonseed oil heated at the b.p. (350°) for 4 hr. (2) Cottonseed oil heated at 320° for 2·5 hr. in the presence of iron, which favours ketonization of the oil.

Considering all tests carried out in this biological investigation, in 268 animals surviving 400 days and over, there were eighteen papillomata and six carcinomata of the forestomach. Although this is a smaller percentage than that which might be expected to follow the administration of a carcinogenic hydrocarbon, it is similar to the percentage of deaths in the human species due to gastric cancer. The present work was undertaken in an attempt to determine the nature of the heated products used in these biological tests. For a preliminary communication of this work see Chalmers (1952).

EXPERIMENTAL AND RESULTS

Apparatus. In the earlier biological experiments (Peacock *et al.* 1953), the oil was heated in a stream of N_2 in a flask fitted with an electrically heated column as described for use in the distillation of fatty esters (Hilditch, 1947). In the present experiments, owing to difficulties due to frothing, the heated column was replaced by a vacuum-jacketed column (3 ft. × 2 in. diam.). A small receiver was inserted immediately above the vacuum jacket, and water formed during the reaction together with a small quantity of liquor smelling strongly of acrolein were run off. The still-head carried a jet for the admission of N_2 in case of frothing: the main receiver was attached directly to the still-head. The path to the receiver and exhaust was about 1 in. wide.

Injection experiments. In addition to the feeding experiments to which reference has been made, several of the cottonseed-oil products were tested by subcutaneous injection, either alone or in solution in tricaprylin, into mice which were 3 months old at the start of the experiment (Table 1). Some of these experiments are still in progress. A number of the animals died within the first year of the experiment, owing to an intercurrent infection. In Expts. 3 and 4 few of the mice lived much longer than 12 months and the results of the tests are therefore inconclusive. There has been no obvious reaction at the site of injection in any of these experiments, although in some of the older animals remote tumours were observed.

Table 1. *Biological tests with heated cottonseed-oil products*

(For experimental details see text.)

Expt. no.	Temperature and duration of heating	Product tested	Mice surviving at (months) 12	15	18	24	Remote tumours observed
1	350° for 4–6 hr.	Crude product and unsaponifiable fraction	14	14	14	7	Mammary adenoma after 18 months. Hepatoma after 24 months. Lung adenocarcinoma after 24 months
2	320° for 2–3 hr. (+iron)	Crude product	13	10	10	4	Lung adenoma after 23 months. Lung adenoma and hepatoma after 27 months. Squamous carcinoma of skin and generalized lymphomatosis of lymph nodes and lungs after 25 months
3	300° for 22–25 hr.	Crude product	8	2	2	0	Lung adenoma after 18 months
4	Peroxidized at room temperature	Crude product	8	4	2	0	Lung adenoma after 22 months

Decarboxylation of cottonseed oil. When testing for the presence of CO_2, the volatile products of pyrolysis carried over in the stream of N_2 were led from the still-head through a wash bottle containing aqueous $Ca(OH)_2$.

Piper *et al.* (1931) described the preparation of certain aliphatic ketones by heating the corresponding fatty acids under reflux at temperatures in the region of 300° in the presence of an iron ring, and it was anticipated that heating cottonseed oil in the presence of iron would result in the formation of ketonic products. In the present experiments, it was found that in the presence of iron filings a vigorous reaction was reached after 0·5 hr. at 340°, after 1·25 hr. at 320° or after 4 hr. at 300°. Iron strips catalysed the decarboxylation reaction, although not quite so effectively as iron filings. The surface area of the iron strips used was equal to that of an iron vessel capable of holding the volume of oil heated. It was considered that the results of this experiment would be comparable with those of Grün, Ulbrich & Krezil (1926) who prepared aliphatic ketones by heating the corresponding fatty acids in an iron vessel and also with ordinary domestic cooking in iron utensils. On heating cottonseed oil alone under the conditions described above, CO_2 was first detected at 345°; in the presence of iron strips it was detected at 305° and in the presence of iron filings at 250°.

Polymerization of cottonseed oil. In the present experiments cottonseed oil was heated at 300° in an atmosphere of N_2. There was a steady increase in viscosity on heating up to 20 hr., between 20 and 25 hr. there was a sharp rise and from 25 to 35 hr. the steady rate of increase was resumed. Fig. 1 shows changes in iodine and free fatty acid values during polymerization.

The polymerization of a number of highly unsaturated oils, including cottonseed oil, by heating at 280° for periods up to 20 hr. has been described by Lips, Promislow & Grace (1953). Under the conditions used by these workers, free fatty acid values remained low and the changes in viscosity and iodine value were lower than those found in the present experiments.

Peroxidation of cottonseed oil. This was done by passing air through a sample of cottonseed oil at room temperature until a high peroxide value was obtained. The sample used for biological test had a peroxide value of 165 and a free fatty acid value of 4·3%.

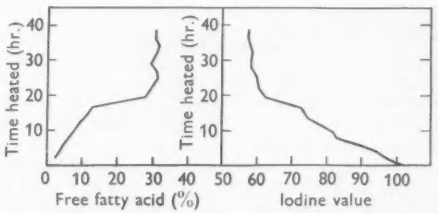

Fig. 1. Iodine and free fatty acid values during polymerization of cottonseed oil.

Fractionation of heated cottonseed oil. The heated product (100 g.) was well stirred with hot acetone (1 l.) and allowed to stand overnight at room temperature. 2N-NaOH was used to separate the free fatty acid (*FFA*) from an ether solution of the acetone-soluble material. Saponification of the remainder of the acetone-soluble material gave acid (*FA* 2) and unsaponifiable (*U* 1) fractions. The acetone-insoluble fraction of the heated product was also saponified giving acid (*FA* 3) and unsaponifiable (*U* 2) fractions. An examination has been made in this way of the composition of the heated cottonseed-oil products used in the biological tests and a comparison made with cottonseed oil polymerized by heating at 300° for 22 hr. The results shown in Table 2 are typical, but there were variations in the results for samples heated on different occasions.

Polymerized cottonseed oil. An examination of the polymeric material in the acidic products has been made, using the method of Cowan, Falkenburg & Teeter (1944). The methyl ester of the acid was distilled in an alembic-type still under reduced pressure and the distillate collected in small fractions. The refractive index of the fractions was determined and plotted against the total percentage distilled up to the mid-point of the fraction. The acetone-insoluble fraction (*FA* 3) of the oil heated at 350° for 4 hr., the free fatty acids (*FFA*) and the fatty acids of the neutral oil (*FA* 2) were examined and found to contain polymeric

Table 2. *Comparison of heated cottonseed-oil products*

(There was a significant production of free fatty acid on polymerizing cottonseed oil at 300°. At 350°, less polymer (acetone-insoluble fraction) was produced and there was a corresponding increase in free fatty acid and unsaponifiable material (acetone-soluble fraction). Heating in the presence of iron filings resulted in the production of acetone-soluble and acetone-insoluble unsaponifiable material. Some of these fractions were ketonic but there was also a large amount of chemically inert material of a tarry nature.)

	Acetone-soluble fraction			Acetone-insoluble fraction	
	Free acids			Free acids,	
Temperature and duration of heating	*FFA* (%)	*FA*2 (%)	Unsaponifiable (%)	*FA*2 (%)	Unsaponifiable (%)
300° for 22 hr.	18	10	1·5	60	2
350° for 4 hr.	48	11	11	18	4
320° for 2·5 hr. (+iron)	2	0	35	0	62

Table 3. *Analysis of methyl esters of heated cottonseed oil*

(The figures in parentheses are percentages of the total heated product. For other details see text.)

		Monomer	Intermediate fraction	Dimer	Non-volatile residue
Fraction of heated product (350° for 4 hr.)					
Free fatty acid, *FFA* (48%)	Yield (%)	40	10	15	30
	Mol.wt.	308		531	1205
	n_D^{30}	1·4260–1·4435		1·4775	1·4925
Neutral oil, *FA*2 (11%)	Yield (%)	50	15	10	25
	Mol.wt.	329		466	1263
	n_D^{30}	1·4325–1·4445		1·4805	1·5020
Acetone-insoluble polymer, *FA*3 (18%)	Yield (%)	15	10	7	67
	Mol.wt.	309		544	934
	n_D^{30}	1·4330–1·4470		1·4780	1·4970
Fraction of heated product (300° for 22 hr.)					
Acetone-insoluble polymer, *FA*3 (60%)	Yield (%)	35	15	15	32
	Mol.wt.			440	932
	n_D^{30}	1·4355–1·4450		1·4750	1·4900

material. The results of these distillations and, for comparative purposes, of the distillation of the *FA*3 fraction of cottonseed oil polymerized by heating at 300° for 22 hr., are shown in Table 3. The acetone-insoluble polymeric fraction of cottonseed oil heated at 350° contained less monomer, less dimer, and more non-volatile polymeric material than the product heated at 300°. The free fatty acid fraction of the oil heated at 350° contained a significant amount of polymeric material and the importance of this is increased by the fact that almost half of this heated product was free fatty acid. The polymerization of the free fatty acid from soy-bean oil has been described by Croston, Tubb, Cowan & Teeter (1952) and presumably in the present case the fatty acid liberated on heating was subsequently polymerized. The neutral-oil fraction was also shown to contain polymeric material.

Cooking tests. Chang, Tchen & Watts (1952) examined the effect of deep-fat frying on the component fatty acids of lard and corn oil. They found that the iodine value of the corn oil dropped from 126·1 to 122·1 after 4 hr. at 180° and that the percentage of conjugated diene showed a slight increase. Changes in fatty acid values were slight, the most marked being shown by linoleic acid, which dropped from 55·0 to 52·2%.

In this laboratory a sample of cottonseed oil which had been used in a local factory in frying potato crisps has been examined by the methods which have been described. In the factory the fat was heated in a deep iron pan to a temperature of about 250°. The sample did not contain any acetone-insoluble polymeric material and the methyl ester of the fatty acids from the neutral oil behaved similarly to the unheated cottonseed oil on distillation in the alembic still, consisting almost entirely of monomer.

In a further experiment potatoes were fried in cottonseed oil in the laboratory. In view of the catalytic effect of iron referred to earlier in this paper, an iron and an aluminium pan were used in parallel experiments. One litre of cottonseed oil was used for each experiment and when the temperature reached 190° the potatoes were added. The temperature dropped to 120° and remained low for about 15 min. Subsequently the temperature rose steadily to 140° at which point the chip potatoes were cooked brown. The time of heating was approx. 30 min. The operation was repeated using the same oil until the total time of heating was 7 hr. An analysis of the oil remaining and of the oil used commercially gave the results shown in Table 4. In the laboratory experiments, the reduction in the iodine value of the used oil was greater than the corresponding increase in free

Table 4. *Effect of potato-frying temperatures on cottonseed oil*

(For details see text.)

Test	Unused cottonseed oil	Cottonseed oil used in frying potatoes		
		Commercially (iron pan)	Laboratory (iron pan)	Laboratory (aluminium pan)
Solubility in acetone	Soluble	Soluble	Soluble	Soluble
Iodine value	104·4	100·1	91·4	89·2
Free fatty acid (%)	None	0·85	0·38	0·33
Peroxide value	1·2	4·7	13·3	9·9
Refractive index (n_D^{30})	1·4680	1·4685	1·4710	1·4720

fatty acid content when compared with oil heated at 300°. In this respect the commercially heated oil occupied an intermediate position. The laboratory heated oil was also oxidized to a greater extent than the commercially heated oil but this was possibly due to the exposure of a relatively greater surface to air. It was noted that the refractive index values varied with the iodine value. Distillation of the fatty acid methyl esters from the neutral fraction of cottonseed oil which had been used commercially in frying potatoes gave no evidence of polymer formation.

DISCUSSION

There is evidence that distillation of the glycerides of saturated fatty acids in the presence of iron converts the fatty acids into the corresponding ketones. Holleman & Koolhaas (1939) distilled coconut oil in the presence of iron and isolated an aliphatic ketone from the distillate. They concluded that small quantities of higher aliphatic ketones are generally formed when food is fried.

There is also evidence that on heating cottonseed oil at 320–340° in the presence of iron the palmitic acid fraction of the oil is converted into palmitone (Chalmers, 1951). In previous experiments, cottonseed oil was distilled in the presence of iron and the solid distillate fractionated by treatment with ethanol. In the higher melting fraction palmitic acid was identified. It was presumed that the fatty acid was first split from the fat and under suitable conditions, e.g. in the presence of iron at about 320°, converted into the corresponding ketone.

Subsequently it was suggested by Hendry, Homer, Rose & Walpole (1951) that ketonization of heated fat might take place with formation of glycidyl esters. Although heated fat products have been tested in this laboratory for the presence of glycidyl esters, with negative results, it is possible that the glycidyl esters are unstable intermediates.

In addition to palmitic acid, cottonseed oil contains oleic and linoleic acids as major components. Isomerization of these acids has been described by Bauer & Krallis (1931) who heated oleic acid in nitrogen at 250° and were able to identify an isomeric acid in the heated product. In the course of a study of the synthesis of triolein, Desnuelle & Naudet (1945) found that '*iso*oleic acid' was formed in amounts varying from 5·1 to 11·8% when oleic acid or triolein was heated at 135–180°.

In a recent paper, Barker, Crawford & Hilditch (1951*a*) have described the interchange of acyl groups in triglycerides containing saturated and unsaturated acids. They heated oleodistearin at 300° in glass or silica vessels and after 10 hr. at this temperature the composition of the mixture was tristearin 25, oleodistearin 54·2, stearodiolein 20·2 and triolein 0·6%. The production of free fatty acid was the only side reaction detected. Acyl migration in the presence of catalysts has been demonstrated by other workers in the case of the glycerides of soy-bean oil or cottonseed oil and similar results were obtained with mixtures of tripalmitin and triolein in the presence of stannous hydroxide at 225° (Norris & Mattil, 1946, 1947) or of tristearin and triolein at 205° in the presence of sodium methoxide (Naudet & Desnuelle, 1947).

The primary change which takes place on heating an oil containing polyethenoid acids is a rearrangement of unconjugated pentadienes to the corresponding conjugated dienes. This change, which thermally takes place at about 300°, can take place at a lower temperature in the presence of catalysts. Following the conjugation stage, polymerization of the oil takes place. The mechanism of polymerization is not as yet completely understood but is a subject that has received considerable study. Thus the kinetics of thermal polymerization of isomers of methyl linoleate have been investigated by Paschke, Jackson & Wheeler (1952) and chemical changes taking place on thermal polymerization of sunflower-seed and linseed oils have been described by Barker, Crawford & Hilditch (1951*b*). Sunflower-seed oil, like cottonseed oil, contains oleic and linoleic acids as major component unsaturated acids, and Hilditch and his co-workers consider that dimerization is effected by a single cross-linking between two carbon atoms in different linoleic acid radicals, intramolecularly in the earlier and intermolecularly in the later stages.

Polymerization increases the flavour stability of oils containing unsaturated acids but has been found to have a deleterious effect on the nutritional value. Lassen, Bacon & Dunn (1949) investigated

the digestibility of polymerized sardine oil incorporated in the diet of rats and found that the polymerized portion of the oil was not absorbed by the gastro-intestinal tract. Further work from a nutritional standpoint has been carried out by Crampton, Common, Farmer, Berryhill & Wiseblatt (1951) and Crampton, Common, Farmer, Wells & Crawford (1953), who fractionated polymerized linseed oil by distillation at low pressure and then by the capacity of the distillate to form urea adducts. When fed to rats, the non-distillable polymeric fraction was largely unabsorbed and the portion of the distillate which did not form urea adducts was found to be toxic. Chemical aspects of this thermal effect on the nutritive value of the polymerized oil have been examined by Wiseblatt, Wells & Common (1953) and by Wells & Common (1953). In the first paper the authors give analytical data, including changes in the fatty acid composition, for linseed oil polymerized by heating at 275° in an atmosphere of CO_2 for various times up to 17 hr. Analytical data are also presented for soy-bean oil heated at various time intervals up to 30 hr. It is suggested that the relationship between the values for polymeric acyl radicals and mean molecular weights of the heated oils indicates the presence of intrapolymers. In passing, it may be mentioned that, unlike in the present experiments with cottonseed oil, little free fatty acid was formed (0·69 % after 30 hr. heating of soy-bean oil). The second paper describes the fractionation of linseed oil polymerized as before by heating in CO_2 at 275° for 12 hr. Distillation of the ethyl esters at low pressure gave a monomeric distillate of which one-fifth was incapable of forming urea adducts. It is suggested, on the basis of fatty acid composition, that this fraction contains cyclic acids. Estimations of acetyl values and of the proportion of terminal groups excluded the presence of hydroxy acids and branched fatty acids.

The possible carcinogenicity of a mixed fat (equal parts of cottonseed oil and whale oil) which had been repeatedly heated at 200–220° was examined by Beck & Peacock (1941). The heated fat was added to an adequate basal diet containing 0·5 % cod liver oil and fed to rats and mice. Within a year signs of avitaminosis-A appeared among the rats and this was followed by gastropapillomatosis in the forestomach. The papillomatosis, however, could be avoided or cured by the addition of raw carrot to the heated fat diet. Beck & Peacock also showed by experiments in which rats were fed a diet supplemented by milk, carrot and heated lard that 'the vitamin-A content of the liver was reduced in proportion to the degree and duration of heating to which the lard was subjected'. They concluded that 'there is present in repeatedly heated fats a factor which interferes in some way with the absorption or metabolism of vitamin A'. It is therefore possible that this condition was caused by the inefficient absorption of vitamin A from the diet due to its retention in the polymeric material of the heated lard which was, to a greater or less extent, unabsorbed.

In the present work consideration was given to the nature of the agent inducing tumours of the forestomach of mice fed a diet containing heated cottonseed oil (Peacock *et al.* 1953). The oil tested was heated (*a*) at 350° for 4 hr. and (*b*) at 320° in the presence of iron for 2·5 hr. These products were fractionated by treatment with acetone and by distillation of the fatty acid after methanolysis in an alembic still. For comparative purposes a sample of cottonseed oil polymerized by heating at 300° for 22 hr. was also examined. In the case of the oil heated at 350°, a high percentage of free fatty acid was formed and this fraction, as well as the acetone-soluble (monomeric acid) and acetone-insoluble (polymeric acid) fractions, contained appreciable amounts of undistillable polymeric material. While the figures given for the oil heated at 320° in the presence of iron indicate that this product is mainly unsaponifiable, some of the samples used in the biological tests contained polymers. Moreover, the unsaponifiable fractions contained, in addition to ketonic material, chemically inert substances of a tarry nature which presumably were decomposition products of polymeric material. In a preliminary account of this work (Chalmers, 1952) the presence of polymers in heated cottonseed-oil products was stressed.

No tumours occurred at the site of injection in the present experiments with cottonseed oil polymerized at 300° or oil heated at 350° for 4 hr. and at 320° for 2·5 hr. in the presence of iron. The experiments with oil polymerized at 300° were inconclusive since many of the animals under test died at a comparatively early age due to an infection. However, in the test with oil heated at 350°, which also contained polymeric material, more animals were used and there was a good survival rate. In this experiment, also, no tumours were found at the site of injection. In the feeding tests, where the route of administration is more satisfactory (Peacock *et al.* 1953), cottonseed oil heated at 350° gave fewer tumours of the forestomach of mice than the product heated at 320° in the presence of iron. It therefore appears that the substance responsible for the induction of tumours of the forestomach of mice fed a heated fat diet is a weak carcinogenic agent. Although there is at present no conclusive evidence concerning the nature of this carcinogen, it is possible that it may be a polymer.

In considering the abnormal products in heated cottonseed oil from the point of view of possible carcinogenicity, reference may be made to the sterol fraction of the oil which, although small in

amount, may be important in view of the carcinogenic activity of cholesterol-rich extracts from biological sources (Hieger, 1947). It is also possible that small quantities of polymeric acyl radicals are formed during potato frying as carried out in the laboratory, although the heated fat did not contain demonstrable amounts of polymeric material. Another possibility would be the formation in heated fats of compounds of a 'cyclic' type, the toxicity of which was described by Crampton *et al.* (1953) in the case of heated linseed oil.

Although there is no evidence associating stearoylethyleneimine with heated fat products, it is interesting that it has been suggested that polymerization plays a part in the action of this synthetic carcinogen in the cell. It has been postulated by Hendry *et al.* (1951) that cytotoxic agents condense intermolecularly within the cell with the formation of polymer units with chemically reactive side chains, which then combine with the protein or nucleoprotein components of the cell. These authors suggested that certain carcinogens acted in the same way and prepared stearoylethyleneimine, which was regarded as a micelle-forming substance capable of reacting with cell components, and showed it to be carcinogenic.

SUMMARY

1. An analysis has been made of cottonseed oil heated (*a*) at 350° for 4 hr. and (*b*) at 320° for 2·5 hr. in the presence of iron. These products, which have been reported to induce tumours of the forestomach of mice, were fractionated by treatment with acetone and by distillation in an alembic-type still. Product (*a*) contained polymeric material in both the acetone-soluble and acetone-insoluble fractions. The major component of (*b*) was unsaponifiable material.

2. Subcutaneous injections into mice of these products and also of polymerized and peroxidized cottonseed oil have not so far, after more than a year, induced any tumours at the site of injection.

3. The changes observed in cottonseed oil used in frying potatoes were comparatively slight and polymerized products were not detected.

The author wishes to thank Dr P. R. Peacock who suggested this investigation. He is also indebted to Prof. T. P. Hilditch for helpful discussion and to Miss R. H. Kennaway and Mr J. McDougall for valuable assistance.

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Studies in Rhodopsin

7. REGENERATION OF RHODOPSIN BY COMMINUTED OX RETINA

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In a previous paper (Collins, Green & Morton, 1953) the regeneration of rhodopsin by eye tissue from frog and rat was studied. It was found that good regeneration *in vitro* could be obtained in a period not very different from that required for dark adaptation in man. The work using frog or rat eyes was rendered difficult and tedious because the absolute amounts of rhodopsin are quite small. It was expected that the use of ox retinas would result in more rapid progress because they are plentiful and because of their size contain at least 20 times as much rhodopsin as frog or rat retinas. Many experimental difficulties were encountered, and it was some time before a reasonable degree of regeneration was obtained with ox retina.

EXPERIMENTAL

Materials

Vitamin-A solution. Crystalline, synthetic vitamin-A alcohol or acetate (Hoffmann-La Roche), was used. The acetate was first saponified before use by N-KOH in ethanol at 53° for 15 min. (see Isler, Roneo, Guex, Hindley, Huber, Dialer & Koffer, 1949). The vitamin-A alcohol was extracted with light petroleum; this solution, after being washed with water, was evaporated to dryness and a solution of Tween 80 (polyoxyethylene derivative of sorbitan mono-oleate; Honeywill and Stein Ltd.) in $CHCl_3$ added. (In the earlier trials the detergent Dispersol A was used unsuccessfully. Tween 80 permits a greater concentration of dispersed vitamin A.) The $CHCl_3$ was removed *in vacuo* and the required volume of water was then added. These operations were carried out in red or dim light and the solution was stored at −78° until required, but even with these precautions it was desirable to prepare fresh solutions frequently. To determine the concentration of vitamin A in such aqueous dispersions, the method described by Collins *et al.* (1953) was used.

The adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), cytochrome *c*, digitonin solution, buffer solution and potash alum solutions were those described by Collins *et al.* (1953).

Pyridoxal phosphate. This was supplied as the calcium salt by Roche Products Ltd.

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METHODS

Preparation of eye tissues. Ox eyes from the local abattoir were transported in light-proof containers and packed with ice. In the laboratory, all work, unless otherwise specified, was carried out in red light. The cornea, lens and vitreous humour were removed from the eye, and then the retina. In some early experiments, whole retinas were used, but the usual procedure was as follows: the retinas were comminuted (using an effective type of homogenizer described by Brendler, 1951) at 0° in whatever medium was to be employed in the subsequent experiment, but this did not include vitamin A. The volume of medium used was 1·5 ml./retina and 2 ml. samples were then taken for the experiment.

Two controls were used in every experiment. (i) Alum was added immediately to a sample (2 ml.), which was then stored in a light-proof tin until required. This sample was used to determine the total rhodopsin present. (ii) Another sample (2 ml.) was exposed to a Photoflood light (Ediswan 82, 230 v) for 1 min. at 6 in. distance, alum was added and the sample was stored until required. This sample was used to determine the trace of rhodopsin remaining after irradiation. (iii) The remainder of the solution was used (as 2 ml. samples) for regeneration experiments. The solution was exposed to a Photoflood lamp for 1 min. and then incubated at 37° for 2 or 3 hr. Vitamin-A or retinene solution was added (either just before irradiation or after the sample was returned to the red light) in sufficient quantity (0·2 ml.) to bring the final concentration to about 8×10^{-4} M.

After the period of incubation, alum was added to all the samples; after 1 hr. the mixtures were centrifuged. The sedimented material was washed with saline solution (0·9% NaCl in water) and re-centrifuged. Then 2 ml. of 1% aqueous digitonin were added and left to extract for 1 hr. The mixture was then centrifuged and the supernatant solution examined in a spectrophotometer (in a 1 cm. cell). The solution was then exposed to a Photoflood lamp for 1 min. and the absorption remeasured.

In many experiments the absorption spectra were measured in the presence of 0·5 M hydroxylamine (Wald & Brown, 1950).

The yield of rhodopsin was given by the difference in the extinction at 500 mμ. before and after exposure to light. A further check could be obtained in the following manner: The absorption between 440 and 600 mμ. consists of that due to rhodopsin, together with irrelevant absorption, the latter largely a result of the solutions being turbid. A plot of log E against log λ for bleached rhodopsin solutions is very nearly a straight line, which is characteristic of turbid solutions. In this spectral region, the absorption due to

retinene follows the same relation. The procedure due to Fog (1952) was therefore applied. The three wavelengths chosen were 430, 500 and 582 mμ., because $\sqrt{(430 \times 582)} = 500$. The general correction equation given by Fog (1952) for any solute with a distinct absorption band distorted by light scattered from suspended matter simplifies for these wavelengths to

$$E_{corr.} = 1{\cdot}215E_{500\ m\mu.} - \sqrt{(E_{430\ m\mu.} \times E_{582\ m\mu.})}.$$

The value of $E_{corr.}$ was usually in good agreement with the value obtained by difference.

The amount of regeneration was often simply expressed as a percentage of the total rhodopsin. However, as both the total rhodopsin and the residual rhodopsin varied somewhat, to facilitate comparison, the yield was more often expressed as 'net percentage regeneration' where

$$\text{percentage regeneration} = \frac{A - B}{T - B} \times 100,$$

and A is the extinction at 500 mμ. due to rhodopsin after regeneration, B is the extinction at 500 mμ. due to the residual rhodopsin after irradiation and storage in alum, and T is the extinction at 500 mμ. due to the total rhodopsin present at the beginning of the experiment.

RESULTS

Preliminary experiments

For a long time it was not here possible to obtain any regeneration with ox-eye tissues. Many different techniques were tried, but without success. At first it was thought that the interval between killing and the use of the eyes at the laboratory was too long, but failures still resulted even when the eyes were kept at 0° until arrival, and with the use of a 'complete' medium (see Collins *et al.* 1953) containing vitamin A.

At this stage it was thought worth while to ascertain whether ox retina was capable of phosphorylation and thus able to effect synthesis.

Cattle retina, kept at 0° until incubated at 37° with a 'complete' medium containing inorganic phosphate labelled with ^{32}P, incorporated the isotope into both ATP and other organic compounds.

In this experiment, the mixture which had been incubated was deproteinized with trichloroacetic acid. The inorganic phosphate was extracted from the aqueous extract by *iso*butanol (5 ml.) after the addition of ammonium molybdate (10%, w/v, 0·5 ml.) and 10N sulphuric acid (0·5 ml.). The extraction took 15 sec. and further extractions (after addition of inorganic phosphate as carrier) resulted in the complete removal of inorganic phosphate and all radioactivity associated with it. The solution was then hydrolysed for 20 min. at 100° and the inorganic phosphate formed on hydrolysis was extracted in the way described above. In two experiments, the easily hydrolysed phosphate (ATP) corresponded with 9 and 5 μg. labile P/ml. of reaction mixture having 730 and 1050 counts/min./ml., respectively. The aqueous phase, which contained the less labile, organically bound phosphate, gave counts/min./ml. of 3150 and 3450, respectively, indicating that 3–4 times as much phosphorus was held in this manner as compared with the phosphorus of ATP. The method outlined here has been independently developed by other workers (see Ennor & Stocken, 1950).

These results indicated clearly that the retina could sustain synthetic processes.

From that point Tween 80 was substituted for the detergent Dispersol A which had been used initially.

Regeneration of rhodopsin using vitamin A

At first the experiments were carried out with retina and choroid and a 'complete' medium. It was found that better results were obtained if choroid was not included. This is quite different from our experience with rat-eye tissue, where the choroid and pigment epithelium is essential for optimum results. (Yield without choroid, 0·006 unit; yield with choroid, 0·019 unit.)

The next stage was to test whether the components of the 'complete' medium were all necessary. In experiments in which ATP was excluded, no fall in the amount of regeneration was observed. Further experiments showed that only vitamin A and phosphate buffer were necessary.

Fig. 1 shows the effect of varying the concentration of vitamin A in the final mixture. The optimum concentration is about $7{-}9 \times 10^{-4}$M, and 20×10^{-4}M gave no increased yield of rhodopsin. Collins, Love & Morton (1952) have shown that isolated rod outer segments from ox contain 22·6 mg. of vitamin A

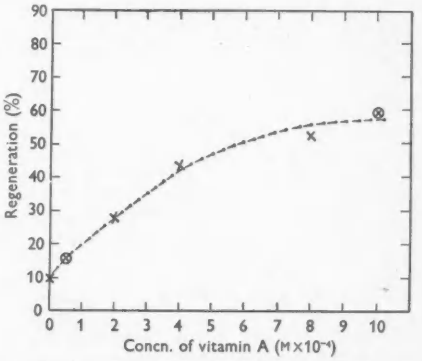

Fig. 1. Regeneration of rhodopsin from ox retina. Regeneration is expressed as a percentage of the initial rhodopsin concentration measured on a separate portion of retinal suspension. The medium was 'complete' (see text) but the vitamin-A concentration was allowed to vary. Regeneration (60%) became independent of concentration of vitamin A above 8×10^{-4}M; (⊗) and (×) refer to separate experiments.

(derived from rhodopsin)/100 g. of wet tissue. If it is assumed that 100 g. wet tissue have a volume of 100 ml., the 22·6 mg./100 g. are equivalent to a $7·9 \times 10^{-4}$M solution of vitamin A. Vitamin-A acetate itself supported regeneration.

The effect of pH was investigated, but as the yield of rhodopsin after regeneration is variable (see below), it proved difficult to obtain a satisfactory curve showing the dependence of the extent of regeneration on pH. However, at pH's less than 7 and greater than 7·6, the yield was much less than in the region 7–7·5.

Fig. 2 shows the rate of regeneration. The average amount of regeneration for twenty-eight samples after 2 hr. incubation was 62 %. The results obtained varied from 48 to 100 %. The figure shows the variable nature of the results, both as to the rate of and the extent of regeneration. This suggests that there must be unidentified factors influencing the results.

The work with frog and rat eyes (Collins *et al.* 1953) showed that regeneration was poor if the eye tissues were not used as soon as possible after the death of the animal, even though a 'complete' medium had been used. Table 1 shows the effect of the lapse of time between comminution and the beginning of incubation. As can be seen, the decrease in the amount of regeneration can be counteracted by DPN. As there was usually a very short interval between comminution and the start of incubation, the addition of DPN was not usually necessary. It was only added when it was desired to take every care that conditions should be optimum.

Table 2 shows the effect of pyridoxal phosphate; in every case its use resulted in an increase in the regeneration of rhodopsin. DPN was added so that its concentration should not be limiting. In all the above experiments the vitamin A was added after the retinal homogenate had been exposed to light and had been returned to a red light, hence regeneration was obtained using *all-trans*-vitamin A.

Regeneration of rhodopsin with retinene

Wald & Brown (1950) obtained good regeneration by flooding a bleached rhodopsin solution with retinene. When this experiment was repeated in this laboratory, no regeneration could be obtained. Subsequent work by Hubbard & Wald (1952*a*, *b*) showed that regeneration from retinene, under the conditions used in their experiments, could only be obtained when the retinene had either been prepared from natural vitamin A or had been exposed to light. The retinene used in this laboratory had been prepared from crystalline, synthetic vitamin-A acetate, and was not unduly exposed to light, although the light was not rigidly excluded as in later experiments.

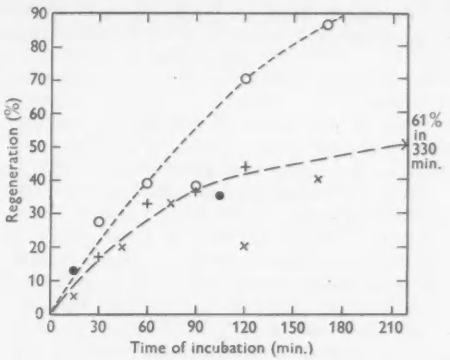

Fig. 2. Regeneration of rhodopsin in relation to time of incubation. Four experiments are shown with ox retina in a medium containing 0·034M phosphate buffer, pH 7·4, and vitamin A. ○○, 7×10^{-4}M; × ×, 7×10^{-4}M; + +, 8×10^{-4}M; ●●●, 4×10^{-4}M. Other experiments in which the final vitamin-A concentration was $8 (\pm 1) \times 10^{-4}$M gave regenerations after 2 hr. incubation not exceeding 60%. The overall average for a series of twenty-eight experiments (2 hr. incubation, final vitamin-A concentration always 4×10^{-4}M) was 62%. The experiment '○○' (above) shows much better results, indicating that the apparent limit of about 60% is due to some unexplained inhibition.

Table 1. *The influence of the freshness of the tissue on the regeneration of rhodopsin*

(Reaction mixture 7×10^{-4}M vitamin-A alcohol, 0·02M phosphate buffer, pH 7·4, and retinal suspension as described in the Experimental section.)

Time elapsing after comminution and the start of incubation (min.)	Expt. no. 1	2	3*
	Net regeneration (%)		
0	62	72	45†
30	44	44	57·5
60	34	30	37·5
90	—	17	52·5
120	—	—	57·5

* In this expt. $2·5 \times 10^{-5}$M DPN was present.
† This low figure is anomalous.

Table 2. *The effect of pyridoxal phosphate on the regeneration of rhodopsin*

(Reaction mixture contained 5×10^{-4}M vitamin-A alcohol, 0·034M phosphate buffer, pH 7·4; 0·02M potassium L-glutamate and $2·5 \times 10^{-5}$M DPN. When added, the pyridoxal phosphate was 2×10^{-3}M.)

Conditions	Expt. no. 1	2	3	4
	Net regeneration (%)			
Without pyridoxal phosphate	32	23	23	23
With pyridoxal phosphate	46	43	43	50

When these results of Wald and his co-workers were published, it became desirable to investigate the effect of light on the present experiments. Table 3 shows that about the same amount of regeneration was obtained when either vitamin A or retinene was used. When either vitamin A or retinene was exposed to light before incubation, the amount of regeneration was increased. Natural vitamin A gave the same amount of regeneration as synthetic *all-trans*-vitamin A.

The experiments recorded in Table 4 were intended to test whether the regeneration occurring in whole retinal suspensions could be reproduced using only the solid portion obtained by centrifuging and washing with 0·9% saline to remove soluble substances. The figures in Table 4 refer to three experiments. In Expt. *A* (8×10^{-4} M retinene) it appears clear that the materials needed for regeneration are contained in the insoluble portion, and that soluble enzymes are not required in the formation of rhodopsin from retinene. This experiment shows the puzzling feature that the regenerations in samples 6 and 7 exceed the control reading for unbleached rhodopsin (sample 5) in the washed centrifugate. This may reflect complications due to an excess of retinene. Expts. *B* and *C* (with 4×10^{-4} M retinene) show that the power to regenerate rhodopsin lies in the insoluble part of the retinal suspension, that water-soluble enzymes are not needed with retinene, and, finally, that retinene is more effective after exposure to light.

Table 3. *The effect of light on the regeneration of rhodopsin from* all-trans-*vitamin-A alcohol or retinene*

(Reaction mixture was $4–8 \times 10^{-4}$ M vitamin-A alcohol or $3–8 \times 10^{-4}$ M retinene and 0·034 M phosphate buffer, pH 7·4. The vitamin A or retinene was either added to the mixture just before or just after irradiation of the rhodopsin prior to regeneration. The figures represent mean percentage of net regeneration (see text), the standard error and, in brackets, the number of experiments.)

	Conditions	
Substance added	Not exposed to light	Exposed to light
Vitamin-A alcohol	55·5±8·8 (8)	81·6±13·0 (5)
Retinene	59·2±12·3 (9)	87·5±8·5 (8)

DISCUSSION

If the results of the present paper are compared with the previous ones (Collins *et al.* 1953) the conditions required for the regeneration of rhodopsin are strikingly different. With frog tissue, the retina and choroid must be incubated in a 'complete' medium including ATP and vitamin-A alcohol. With rat, both the retina and choroid are required, but the medium need only contain vitamin A and a buffer (phosphate, pH 7·4). With ox, only the retina is required along with vitamin A and a buffer (pH 7·4). These differences are presumably correlated with the dependence of the visual cells on a retinal or a choroidal circulation, and on whether the tissue is poorly or richly supplied with metabolites. Ox retina, isolated by the same techniques as were used in the regeneration experiments, is capable of phosphorylation, and is thus capable of supporting many synthetic processes. Therefore these interspecies differences do not necessarily imply any fundamental differences in the mechanism of

Table 4. *Regeneration of ox rhodopsin by retinene. Comparison between whole-retinal suspensions and the solid obtained from suspensions after centrifugation*

(Reaction mixture was retinene (concentration shown below) and 0·034 M phosphate buffer, pH 7·4. In samples 1 to 3 the whole suspension was used, in samples 4 to 7 the retinal suspension was centrifuged and the residue washed with 0·9% (w/v) NaCl. The washed residue was finally suspended in the phosphate buffer. The retinene was added just before or just after irradiation of the rhodopsin prior to incubation.)

Sample no.	Experiment ... ... Final concentration of retinene ($\times 10^{-4}$ M) ...	*A* 8	*B* 4 ($E_{1\,cm.}$ at 500 mμ.)	*C* 4
1	Residual rhodopsin at start	0·026	0·011	0·018
2	Regenerated rhodopsin; *retinene exposed to light	0·245	0·280	0·192
3	Regenerated rhodopsin; retinene not exposed to light	0·250	0·268	0·123
4	Residual rhodopsin at start	0·015	0·003	0·022
5	Rhodopsin before bleaching	0·189	0·237	0·239
6	Regenerated rhodopsin; *retinene exposed to light	0·256	0·196	0·173
7	Regenerated rhodopsin; retinene not exposed to light	0·272	0·140	0·059

* Retinene added before bleaching, i.e. it could have been isomerized by the light.

regeneration. The retina and choroid of frog may be poorly supplied with metabolites and coenzymes, while the choroid of the rat may be well supplied. The fact that ox retina appears to be well supplied with metabolites and coenzymes increases the experimental difficulties, as it is difficult to determine which metabolites and coenzymes are directly needed for the regeneration of rhodopsin.

In the case of pyridoxal phosphate, only a small effect would be expected if it were added to a comminuted retinal suspension already containing a nearly adequate amount. The fact that pyridoxal phosphate has such a significant effect suggests that it is directly concerned in the regeneration of rhodopsin, and not with an indirect process.

The results were quite variable and, even with pyridoxal phosphate, 100% regeneration was not obtained, although on other occasions 100% regeneration was obtained without pyridoxal phosphate. Nor did the use of a 'complete' medium result in consistently better results. Therefore all the factors necessary for the regeneration of rhodopsin are not yet known.

Hubbard & Wald (1952*a*, *b*) have shown that light has an important effect on regeneration from retinene. They found that *all-trans*-retinene added to a bleached rhodopsin solution produced no regeneration, but if the retinene had previously been exposed to a bright light, as much as 85% regeneration resulted. The same result was obtained if the retinene had been prepared from natural vitamin-A esters. They suggested that only a *cis* isomer of retinene was effective in regeneration. Hubbard & Wald (1951) obtained good regeneration using the system, vitamin A (as the alcohol, prepared from a fish-liver oil), DPN, crystalline alcohol dehydrogenase, and opsin. Opsin was obtained from rod outer segments which had been hardened in alum, washed with a neutral buffer and finally extracted with aqueous digitonin. This digitonin solution would contain, among other things, the protein moiety of rhodopsin. Using this system, they obtained up to 65% regeneration in 9 hr. or more. Later, Hubbard & Wald (1952*a*, *b*) tested *all-trans*-vitamin A and an isomer, neovitamin A; both were inactive. According to current theories (Pauling, 1949), only four stereoisomers of vitamin A or retinene are possible. Hubbard, Gregerman & Wald (1953) have now prepared five 'isomers' of retinene. Only 'neoretinene *b*' gave rhodopsin on incubation with the system described above. '*iso*Retinene *a*' yielded a 'rhodopsin' with $\lambda_{max.}$ displaced to 487 mμ. The other forms of retinene were inactive.

In the simple system used by Wald and his co-workers, only specific forms of vitamin A and retinene were capable of supporting regeneration. In the present work a more complex system was used and regeneration was obtained from *all-trans*-vitamin A or retinene. The simplest explanation is that there is an enzyme capable of catalysing the change of *all-trans*-vitamin A or retinene to the active form, which then combines with opsin. The fact that irradiation of the vitamin A or retinene prior to incubation increased the amount of regeneration would suggest that the amount of this hypothetical enzyme might be a limiting factor in the retinal suspension used in the present work. It cannot be stated whether this enzyme would act on *all-trans*-vitamin A or its aldehyde, as they are equally effective in regeneration, perhaps because the equilibrium catalysed by alcohol dehydrogenase is reached quickly, in which case it would be immaterial whether the alcohol or aldehyde were used.

The course of regeneration can be formulated as follows: The initial step is almost certainly

$$\text{vitamin A} + \text{oxidized DPN} \rightleftharpoons \text{retinene} + \text{reduced DPN}. \quad (1)$$
(alcohol dehydrogenase)

The next reaction would be the conversion of retinene into active retinene. The nature of the active form is at present unknown, but there seem to be at least three possibilities. (i) It could be a *cis* isomer, as first suggested by Hubbard & Wald (1952*a*). There appear to be five 'isomers' of retinene, in apparent conflict with Pauling's (1949) theory; one or more of these could be non-stereoisomeric (e.g. a dimer), but the active form could still be a *cis* isomer. (ii) The active form could contain an α-ionone ring, as suggested by Hubbard *et al.* (1953). (iii) The active form could be a dimer of retinene. Hence, the second step can be written as follows:

$$\text{retinene} \rightarrow \text{'active retinene'} \text{ (isomerase or dimerase)}. \quad (2)$$

The work reported in this paper suggests that the isomerase or dimerase is insoluble in water and is inactivated by alum.

Wald and his co-workers have shown that 'active retinene' reacts spontaneously with opsin to form rhodopsin. At present the details of this reaction are unknown, but two important facts are relevant. Wald & Brown (1952) have shown that sulphydryl groups are liberated on irradiation of rhodopsin, and that retinene will combine with sulphydryl compounds in slightly acid or neutral media. Collins (1953) has shown that the first stable product after irradiating rhodopsin is indicator yellow (first described by Lythgoe, 1937), and that this material is not an artifact as suggested by Ball, Collins, Morton & Stubbs (1948) and by Wald (1951). Retinene has been shown to combine with primary amines in an alkaline medium to form Schiff's bases, which have absorption spectra closely resembling that of indicator yellow (Ball, Collins, Dalvi & Morton, 1949; Collins & Morton, 1950). The above evidence suggests that retinene can combine with sulphydryl groups at pH 7 and not with amino

groups, but that after irradiating rhodopsin, the retinene is combined with an amino group and not a sulphydryl group. A possible mechanism is as follows:

$$\left.\begin{array}{l}\text{'active retinene'} + \text{opsin} \rightarrow \text{retinene-}S\text{-opsin},\\ \text{retinene-}S\text{-opsin} \rightarrow \text{retinene-}N\text{-opsin (intramolecular).}\end{array}\right\} \quad (3)$$

Rhodopsin could be the compound with either a retinene-to-sulphur bond or a retinene-to-nitrogen bond. However, the fact that compounds of retinene and sulphydryl compounds absorb at short wavelengths (about 330 mμ.), while the compounds of retinene and a primary amine can absorb at long wavelengths (440 mμ.), and even at 500 mμ. under certain conditions (Collins, 1953), strongly suggests that rhodopsin contains a retinene-to-nitrogen bond.

The role of pyridoxal phosphate is not implicit in the foregoing. If it be assumed that it is acting as a coenzyme of transamination, its function may be to maintain an essential amino group, present in opsin. It is even conceivable that pyridoxal phosphate may be transformed into pyridoxamine phosphate, which may be part of the opsin molecule.

SUMMARY

1. The regeneration of rhodopsin has been studied with comminuted ox retina. The reaction mixture need only contain *all-trans*-vitamin-A alcohol and phosphate buffer (pH 7·4). The average amount after 2 hr. at 37° was 62 %, but the results were variable and ranged from 48 to 100 %.

2. Pyridoxal phosphate was found to increase the extent of regeneration.

3. The effect of light on the vitamin A or retinene used showed that vitamin A and retinene were equally effective and that prior exposure to light resulted in increased regeneration in both cases.

4. The implications of these results are discussed in connexion with current theories of the mechanism of the regeneration of rhodopsin.

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Comparative Detoxication

2. GLUCOSIDE FORMATION FROM PHENOLS IN LOCUSTS

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It has recently been shown that locusts, like mammals, can acetylate aromatic amino compounds (Myers & Smith, 1953*a*) and form substituted hippuric acids from aromatic acids (Friedler & Smith, 1953).

When phenols are absorbed by mammals, a considerable proportion of the dose is eliminated in the urine as ethereal sulphates and glucosiduronic acids (glucuronides) (Williams, 1947). With large doses of phenols glucosiduronic acid formation is usually predominant (Bray, Humphris, Thorpe, White & Wood, 1952). Glucosiduronic acids are also formed in birds (Sperber, 1947) and frogs can convert menthol into menthylglucosiduronic acid (Schmid, 1936).

Apart from a preliminary report on part of the present work (Myers & Smith, 1953*b*), the only reference to the conjugation of an ingested phenol

by an insect appears to be Zukel's (1944) histochemical detection of a 'leucothionol conjugate' in the malpighian tubules of cockroaches poisoned with phenothiazine. The chemical nature of this conjugate, however, was not established. In cockroaches, DDT (1:1:1-trichloro-2:2-di(*p*-chlorophenyl)ethane) is converted into a water-soluble metabolite of unknown constitution which may also be a conjugate of some kind (Butts, Chang, Christensen & Wang, 1953). The object of the present work was to find out whether glucosiduronic acids were formed from phenols in locusts.

It will be shown that the conjugation of *m*-aminophenol and quinol does occur in the locust to a limited extent, but that the conjugates are not the glucosiduronic acid derivatives into which these phenols are converted in vertebrates (Williams, 1943; Garton & Williams, 1949). It was not possible to isolate the conjugates and identify them by classical methods, but chromatographic and other evidence strongly suggests that they are the β-glucosides of the phenols administered.

MATERIALS AND METHODS

Locusts. The 5th-instar hoppers, or immature adults, of *Locusta migratoria* were obtained from the Anti-Locust Research Centre and kept as previously described (Myers & Smith, 1953*a*) on a diet of fresh grass.

Reference compounds. *m*-Aminophenyl-β-D-glucoside, m.p. 139–140°, $[\alpha]_D - 67°$ in water (*c*, 1), was synthesized according to Latham, May & Mosettig (1950). Arbutin (*p*-hydroxyphenyl-β-D-glucoside), m.p. 199°, $[\alpha]_D - 60°$ in water (*c*, 1), was purchased (British Drug Houses Ltd.). The glucosiduronic acids of 7- and 8-hydroxyquinoline and *m*-aminophenol were biosynthetic samples previously prepared in this laboratory (Smith, 1953; Robinson, Smith & Williams, 1953*a*; Williams, 1943). Quinolyl-8-sulphuric acid was prepared by the general method of Burkhardt & Lapworth (1926) using chlorosulphonic acid.

Administration of compounds. The phenols, dissolved in 0·01–0·02 ml. of water, were injected into the thorax at the root of the hind leg. An Agla micrometer syringe (Burroughs Wellcome and Co.) with a no. 27 needle was used.

Preparation of extracts. The dry excreta were ground roughly in a mortar to break up the pellets and allowed to stand for 15 min. in water with occasional stirring. It was usually convenient to use 1–5 ml. of water for the faeces from one locust, depending on the quantity excreted. For the quantitative experiments the mixture was centrifuged or filtered and portions of the clear solution were withdrawn for analysis. In the qualitative experiments the aqueous extracts were filtered under suction, evaporated to small bulk *in vacuo*; absolute ethanol was then added till precipitation of inorganic material and urates was complete. The filtered ethanolic solution was concentrated *in vacuo* before chromatography.

Paper chromatography. Downward chromatograms were run as previously described (e.g. Smith, Smithies & Williams, 1953). The solvents used were: *A*, butanol:acetic acid:water (4:1:5, v/v); *B*, butanol:benzene:ammonia solution (sp.gr. 0·880) (5:2:2, v/v); *C*, pyridine:ethyl acetate:water (1:2:2, v/v); *D*, butanol:water. In all cases the layer containing least water was used. R_F values were not exactly reproducible in the different tanks, but the general pattern and order of separation were consistent, and R_F values are quoted only to illustrate the degree of separation obtained between spots. All chromatographic identifications were made by comparison with an authentic sample run at the same time on the same paper, and were supported by colour reactions and spectrophotometric observations when sufficient material was available.

In general, when the crude faecal extracts, or extracts of normal faeces with added reference compounds, were chromatographed, the spots obtained had smaller R_F values than those given by pure solutions of reference compounds applied alongside. This appeared to be due to a slowing down of the solvent front as it passed the heavy deposit of irrelevant material in the spot of dried extract. It was necessary, therefore, to separate the metabolites on a preliminary chromatogram, to elute areas on the paper giving the appropriate colour tests and to re-chromatograph the eluates. These eluates, which contained less irrelevant material, yielded satisfactory chromatograms with no distortion of the front and were suitable for comparison with the reference compounds.

Paper ionophoresis. An apparatus essentially the same as that of Durrum (1950) was used, the whole assembly being set up on the grid of a large desiccator containing water to maintain humidity. A strip of Whatman no. 3 paper, 8 × 30 cm., was supported over a glass rail and dipped into the troughs containing the electrodes. The paper was first dipped into the buffer solution (Britton & Robinson, 1931), freed of excess of fluid by blotting with a sheet of dry filter paper, and hung over the rail dipping into the buffer in the troughs. Spots of the solutions under examination were applied to the centre of the paper and a potential of 350 v was applied to the electrodes from dry cells. A current of 6–10 mA was drawn. After 2 hr. the paper was removed and allowed to dry before being sprayed with reagents to reveal the spots.

Detecting reagents. Reducing sugars were detected by the naphthoresorcinol reagent (Partridge, 1948) or by *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950). Aromatic amino compounds were located by diazotizing and coupling. A spray of 1% (w/v) aqueous HNO_2 followed 5 min. later by one of 1% (w/v) β-naphthol in N-NaOH gave red colours. Quinol and its glucoside, arbutin, were detected by a spray of 0·1% (w/v) tetra-azotized di-*o*-anisidine in 0·5 N-$NaHCO_3$, which gave brown-purple colours.

Glucosiduronic acid was measured by Paul's (1951) modification of the naphthoresorcinol reaction and *m*-aminophenol by the method used by Smith & Williams (1949) for aniline.

Absorption spectra were determined in a Unicam S.P. 500 Spectrophotometer.

RESULTS

Attempted detection of glucosiduronide formation

Quantitative estimations

The excreta of locusts feeding on grass contained a considerable amount of reducing carbohydrate material and gave a strong qualitative naphthoresorcinol reaction for uronic acids. Faecal pellets

had an apparent glucosiduronic acid content of 6 mg./g. which was the same as that obtained on a sample of air-dried grass. Separate examination of the gritty, malpighian excretion, hand picked from excreta, showed that this too gave a naphthoresorcinol colour which corresponded to 13 mg. glucosiduronic acid/g. These amounts were such that any small increase of glucosiduronic acid arising from doses of test compounds would not be detectable. Since the carbohydrate material was not soluble in ether, attempts were made to detect the ether-soluble glucosiduronic acids of borneol and (−)-menthol which are known to be formed in many of the higher animals (Williams, 1947).

Aqueous extracts of locust excreta were acidified with 2N-H_2SO_4 and continuously extracted with ether for 2 hr. The residue after evaporation of the ether was dissolved in 0·1N-NaOH and its glucosiduronic acid content determined. Excreta from locusts eating 1 g. of grass daily gave only 0·1 mg. of apparently ether-soluble glucosiduronic acid/locust/day; 90% of (+)-*iso*menthylglucosiduronic acid (Williams, 1940) added to faeces could be accounted for in recovery experiments. When, however, 2 mg. of borneol or (−)-menthol in concentrated ethanolic solution were injected into the locusts, no rise in the excretion of ether-soluble glucosiduronic acids was found. It appeared therefore that little, if any, of these two compounds was conjugated with glucosiduronic acid.

Qualitative experiments

Compounds selected for preliminary experiments were *m*-aminophenol and 7- and 8-hydroxyquinolines, since the quinolyl glucosiduronic acids are readily detected by their fluorescence in ultraviolet light, and *m*-aminophenylglucosiduronic acid gives an intense red colour with the diazo reagents. These colour reactions enable small amounts of the glucosiduronic acids to be easily located on paper chromatograms.

7-*Hydroxyquinoline.* Two locusts were injected in the thorax with 1 mg. of the compound dissolved in 0·01 ml. of water containing the minimum amount of hydrochloric acid for complete solution (pH about 5) and the faeces were collected during the next 24 hr. The excreta were ground with 2 ml. of 2% (w/v) aqueous sodium bicarbonate and filtered. The filtrate was neutralized with dilute hydrochloric acid and ethanol was added to precipitate inorganic salts. After filtration, the ethanol was evaporated to small bulk and the solution chromatographed on paper using solvent mixture *B* and reference spots of authentic quinolyl-7-glucosiduronic acid and 7-hydroxyquinoline. No spots were found on this chromatogram corresponding with the R_F or fluorescence of the glucosiduronic acid, though two other, faster-moving, unidentified spots (R_F 0·1 and 0·2) were present which fluoresced blue in ultraviolet light.

8-*Hydroxyquinoline.* A similar experiment with 8-hydroxyquinoline yielded a chromatogram on which two spots were present. One, which moved faster (R_F 0·26) than quinolyl-8-glucosiduronic acid (R_F 0·07), fluoresced blue in alkaline and acid solutions and was not identified; the other corresponded approximately (R_F 0·36) to quinolyl-8-sulphuric acid (R_F 0·33) and had a similar blue fluorescence in ultraviolet light.

m-*Aminophenol.* A similar experiment with *m*-aminophenol yielded an extract which was chromatographed on paper in solvent mixture *A*. In addition to a spot corresponding to *m*-aminophenol, there was present a diazotizable substance which had an R_F greater than that of *m*-aminophenylglucosiduronic acid (Table 2). No spot was found which corresponded to that of the glucosiduronic acid though some diazotizable material was left at the starting point.

Separation of the m-*aminophenol conjugate*

Fifty immature adult locusts were each injected on two successive days with 1 mg. of *m*-aminophenol dissolved in 0·01 ml. of water containing the minimum amount of hydrochloric acid for solution (pH about 4). The faeces were collected on the third day, ground in a mortar and extracted twice with 10 ml. of boiling water. The filtrate from the grass residues was concentrated *in vacuo* to small bulk and absolute ethanol was added to precipitate inorganic salts. The pink precipitate was removed and the yellow filtrate concentrated and applied from edge to edge near the end of a sheet of Whatman no. 1 paper as a zone 2 × 20 cm. The paper was irrigated overnight with the top layer of solvent mixture *A* and, after drying, a strip was cut from one edge and sprayed with the diazotizing reagents. Apart from some residual reacting material at the origin, two main bands were present. Unchanged *m*-aminophenol, R_F 0·7, was eluted chromatographically with 0·1N-HCl and its identity confirmed by its ultraviolet absorption (Table 1) and behaviour on paper chromatograms (Table 2). The metabolite having R_F 0·3 was eluted chromatographically with water and purified on another large-scale paper chromatogram in solvent mixture *B*. The diazotizable zone was eluted from the dried paper with water, and after hydrolysis of a small portion with N-HCl for 1 hr. at 100°, it was estimated that this fraction contained 5 mg. of combined *m*-aminophenol. Thus about 5% of the dose could be separated as this conjugate. When run on paper chromatograms it had the same R_F values as authentic *m*-aminophenyl-β-glucoside. A solution of the conjugate gave $[\alpha]_D^{20} - 86° \pm 6°$ in water (*c*, 0·174, estimated from *m*-aminophenol formed on

Table 1. *Ultraviolet absorption values for* m-*aminophenol and its glucoside*

	In 0·1 N-HCl			In 0·1 N-NaOH		
	$\lambda_{max.}$ (mμ.)	$E_{1\,cm.}$	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ.)	$E_{1\,cm.}$	$\epsilon_{max.}$
m-Aminophenyl glucoside						
From locust excreta	265	0·79*	—	283	1·28*	—
Reference sample	266	—	770	283	—	1600
				230–235		5800
m-Aminophenol						
From glucoside of locust excreta	270	1·79*	—	290	2·31*	—
Unchanged, from locust excreta	270	0·52	—	291	0·70	—
Reference sample	270	—	1800	291	—	2900

* Values obtained at equivalent concentrations.

Table 2. R_F *values of some phenols and their conjugates*

(Solvent mixtures: *A*, butanol:acetic acid:water (4:1:5, v/v); *B*, butanol:benzene:ammonia, sp.gr. 0·880 (5:2:2, v/v); *C*, pyridine:ethyl acetate:water (1:2:2, v/v); *D*, butanol:water. Run on Whatman no. 4 paper until fronts had moved about 12 in.)

	R_F value in solvent mixture			
	A	*B*	*C*	*D*
7-Quinolinol	—	0·75	—	—
Quinolyl-7-glucosiduronic acid	—	0·03	—	—
8-Quinolinol	—	0·82	—	—
Quinolyl-8-glucosiduronic acid	—	0·07	—	—
Quinolyl-8-sulphuric acid	—	0·33	—	—
m-Aminophenol	0·85	0·75	0·95	0·75
m-Aminophenylglucoside	0·43	0·17	0·80	0·15
m-Aminophenylglucosiduronic acid	0·21	0·0	0·70	0·0
Quinol	0·95	0·90	1·0	0·85
p-Hydroxyphenyl glucoside	0·20	0·43	0·92	0·30

Table 3. *Ionophoresis on paper of* m-*aminophenol and its conjugates*

(The signs −, +, 0 indicate movement towards cathode, towards anode or no movement, respectively, under the conditions described in the text.)

pH	Excreted conjugate	*m*-Aminophenyl-glucosiduronic acid	*m*-Aminophenyl-sulphate	*m*-Aminophenyl-glucoside	*m*-Aminophenol
1·0	−	−	+	−	−
2·5	−	−	+	−	−
2·9	−	0	+	−	−
3·1	−	+	+	−	−
3·6	−	+	+	−	−
6·0	−	+	+	−	−
8·0	−	+	+	−	−
12·0	−	+	+	−	+

hydrolysis and assuming that the conjugate is *m*-aminophenylglucoside).

The conjugate was obtained also from the excreta of 5th-instar hoppers and immature adults after oral administration and from hoppers to which *m*-aminophenol had been applied topically in acetone solution.

Paper ionophoresis. The directions of movement of the metabolite and reference compounds in buffers of different pH is shown in Table 3. In 2 hr. the displacement of the spots was of the order of 1–1·5 cm., except in the case of the ethereal sulphate which separated rapidly from the other spots at all pH values to a distance of 4–5 cm. Some extracts of locust faeces prepared as described above also contained a trace of a diazotizable substance which moved to the anode like the ethereal sulphate. The nature of this compound, which was not present in all experiments, is being investigated. The behaviour of the major metabolite was similar to that of *m*-aminophenyl-β-glucoside in that it never acquired a negative charge in the pH range 1–12 and therefore contained no acid group. The small migration to the cathode in alkaline solution which occurred to the same extent with conjugate or glucoside, and also with *m*-aminophenol except at pH 12, was presumably due to electro-osmotic effects, since it was found that a spot of glucose was similarly displaced a small way towards the cathode. In the acid region, where the amino group was positively

charged, migration to the cathode was in the expected direction.

Ultraviolet absorption spectra. Spectra of *m*-aminophenyl-β-D-glucoside are plotted in Fig. 1 together with spectra of arbitrary dilutions of a concentrate of the conjugate. On hydrolysis of the solutions of the conjugate by N-HCl for 20 min. at 100°, the absorption maxima shifted to wavelengths characteristic of *m*-aminophenol (Table 1).

Acid hydrolysis of the conjugate. A solution (2 ml.) of the metabolite containing the equivalent of 3 mg. of *m*-aminophenol was made 0·5N with HCl and heated at 100° for 30 min. Spots of the hydrolysate were chromatographed in solvent mixture *A* with reference spots of *m*-aminophenol, glucosiduronic acid, glucurone, glucose, fructose, xylose, arabinose, rhamnose and 6-deoxyglucose. *m*-Aminophenol was detected but no unhydrolysed conjugate was present. An intense spot corresponding to glucose was found together with a weak spot corresponding to xylose. Weak xylose and glucose spots were, however, also found in control extracts made from the excreta of locusts which had not received *m*-aminophenol. Treatment of the bulk of the hydrolysate with phenylhydrazine acetate yielded yellow crystals having the form of glucosazone, but insufficient in amount for further identification.

Enzymic hydrolysis. A drop of a solution of the conjugate containing the equivalent of about 50 μg. of *m*-aminophenol was incubated with 1 mg. of emulsin (British Drug Houses Ltd.) for 2 hr. at 37°.

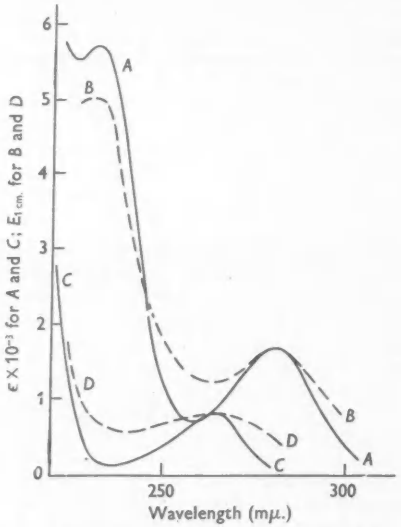

Fig. 1. Absorption spectra of *m*-aminophenylglucoside (*A*, in 0·1N-NaOH, *C*, in 0·1N-HCl) and of extract from locust faeces (*B*, in 0·1N-NaOH, *D* in 0·1N-HCl).

Chromatography in four solvents showed that complete hydrolysis to *m*-aminophenol had occurred. *m*-Aminophenylglucosiduronide was not hydrolysed by this treatment. On the other hand, locust crop fluid, which readily hydrolysed *m*-aminophenylglucosiduronic acid (cf. Robinson *et al.* 1953*a*), had little effect on the conjugate, and after 24 hr. incubation and subsequent chromatography most of the diazotizable material was still present as conjugate.

The conjugation of quinol in the locust

Twenty immature adult locusts each received 0·3 mg. of quinol dissolved in 0·005 ml. of water by injection into the thorax. The dose was repeated the next day and 2 days' excreta were collected and extracted with water and ethanol as described for *m*-aminophenol. The extract was separated on a large-scale paper chromatogram as above, using solvent mixture *B* and Whatman no. 4 paper in a 5 hr. run. In addition to unchanged quinol, moving with the solvent front, and material remaining at the point of application, substances with R_F 0·1, and 0·2 were detected. Corresponding zones were eluted with water and examined chromatographically. The substance with R_F 0·1 appeared to be a conjugate as it yielded quinol on hydrolysis and it is still under investigation. The substance with R_F 0·2 was chromatographically identical with *p*-hydroxyphenyl-β-D-glucoside in the four solvents used (Table 2).

The bulk of the material from the elution was hydrolysed and chromatographed overnight in the same way as the conjugate of *m*-aminophenol. Quinol and glucose with a trace of xylose were detected.

Toxicity of quinol and arbutin. Groups of four adult locusts which had been maintained on a liberal diet of fresh grass were injected in the thorax with varying amounts of quinol in 5% (w/v) aqueous solutions. All locusts in the group injected with 0·5 mg. were dead within 24 hr.; those receiving 0·1 mg. showed no ill effects, while half of those receiving 0·3 mg. were dead in 24 hr. and all showed signs of distress on injection. Similar groups which were injected with increasing volumes of a 10% (w/v) aqueous solution of arbutin, up to 8 mg. arbutin/locust, showed no ill effects and sustained no deaths within 24 hr. Arbutin (4 mg.) administered orally as previously described (Myers & Smith, 1953*a*) had no apparent effect on the locusts.

DISCUSSION

The above results show that the locust eliminates roughly 5% of a 1 mg. dose of *m*-aminophenol in a conjugated form. This conjugate does not behave on paper chromatograms like *m*-aminophenylglucosiduronic acid and electrophoretic experiments show that the molecule has no acid function. Moreover, on hydrolysis, no glucosiduronic acid nor

glucurone was detected, but only glucose. There was the possibility that the sugar part of the molecule was a glucose oligosaccharide, but the formation of a similar conjugate from quinol with the same chromatographic behaviour as *p*-hydroxyphenyl-β-glucoside supports the idea that the metabolites are in fact simple glucosides. That these have the β-configuration is indicated by the ready hydrolysis by emulsin and the optical rotation. This conjugation is, moreover, a genuine detoxication since, in our locusts, injection of arbutin equivalent to 3 mg. of quinol had no apparent effect while one-fifth of this amount of free quinol was rapidly fatal.

The formation of glucosides is well known in plants where the natural glycosides have been thought to be waste products of the plants' metabolism detoxified by conjugation with various sugars. Foreign organic compounds, e.g. chloral, which can give rise to glucosiduronates in animals, form glucosides or gentiobiosides in plants (see Pigman & Goepp, 1948).

The glucosidic detoxication is, however, unusual in the animal kingdom though some observations have been made of the presence of glucosides as normal constituents of insects. Hollande (1913) considered that the pigmented granules in the larvae of the beetle *Cionus olens* were a glucoside which was probably the anthocyanin of the food deposited unchanged in the fat body. Carminic acid, from the cochineal bug, is a sugar derivative but the nature of the sugar is uncertain (Dimroth & Kammerer, 1920). The pigments of aphids can be obtained as water-soluble precursors, protoaphins, which have been shown to be the glucosides of the aphins (Brown, Ekstrand, Johnson, Macdonald & Todd, 1952). In the light of the present work it seems possible that the protoaphins may be detoxication products of the phenolic aphins and that the glucose detoxication mechanism may not be confined to the locust.

On the other hand, some insects are said to be capable of digesting and metabolizing glucosides in their food and salicin (*o*-hydroxymethylphenyl-glucoside) is excreted as salicyl aldehyde or salicylic acid by a variety of insects (Wain, 1943; Hollande, 1909). The locust has a very active glucosiduronic acid-splitting enzyme in its crop fluid (Robinson *et al.* 1953*a*) and this factor may have influenced the use of glucose rather than glucosiduronic acid for detoxication. If any of this enzyme passes into the mid gut, where the malpighian tubes open just anterior to the ileoventricular sphincter, it might hydrolyse any glucosiduronic acid excreted by the tubules. Elimination of glucosiduronic acids would not, therefore, be an efficient mode of detoxication in this insect.

It is also of interest that glucosides are neutral products. Highly ionized products, such as glucosiduronic acids, ethereal sulphates, or hippuric acids, are characteristic of vertebrate detoxication (cf. Robinson, Smith & Williams, 1953*b*).

SUMMARY

1. The conjugation of hydroxy compounds by *Locusta migratoria* has been investigated by paper chromatographic and other methods.

2. No glucosiduronic acid conjugates were produced by (−)-menthol, borneol, 7- or 8-hydroxyquinoline, or *m*-aminophenol.

3. *m*-Aminophenol and quinol were converted, in part, into compounds having the properties of the corresponding β-D-glucoside.

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On the Flow of Certain Pathological Human Synovial Effusions through Narrow Tubes

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Viscosity-concentration relations in both human and bovine synovial fluids have been the subject of considerable attention. (For a recent summary see Frey-Wyssling (1952).) But, although it is generally admitted that most samples are not simple Newtonian liquids, few authors have studied the relationship between rate of shear ($\dot{\gamma}$) and stress (τ). The only notable exceptions are Ogston & Stanier (1950) who, using non-pathological bovine fluids, found an approximate empirical linear relation between $\eta_{rel.} - \eta_{rel.\infty}$ and $1/(\dot{\gamma}+a)$, where η_{rel} is relative viscosity, $\eta_{rel.\infty}$ is relative viscosity at high shear-rates and a is a constant. To substantiate this relationship only a few curves are shown. This equation is very similar to that proposed by Goodeve & Whitfield (1938) for systems which they describe as thixotropic, though some other rheologists disagree with this use of the term. This equation has, in its turn, been shown to be related to the Bingham equation (see Scott Blair, 1949). A similar relation has also very recently been discussed by Prins & Hermans (1953). Since it is established that synovial effusions are in many cases not simple fluids having viscosities independent of rate of shear, it is clearly desirable further to investigate their viscous anomalies, with a view to establishing the number and nature of the parameters needed to describe their behaviour adequately. This is the purpose of the present work.

It has long been known that many colloidal solutions, which, when dilute, obey Newton's law, $\tau/\dot{\gamma} = \eta = \text{const.}$, show, for higher concentrations, a decrease in the ratio $\tau/\dot{\gamma}$ as stress and rate of shear rise; and various empirical functions, such as power laws, have been proposed to fit the facts. The whole subject is admirably summarized by Reiner (1949). Much earlier, however, Ostwald (1925) pointed out that it is not possible that $\tau/\dot{\gamma}$ should continue to decrease indefinitely with increasing shear rate, since the viscosity will presumably never fall below that of the pure solvent (normally water). For this reason he postulated a curve which, at lower stresses, follows a power law but which later inflects and, at higher stresses becomes linear (the 'Laminarast') and is directed towards the origin. Few such curves have appeared in the experimental literature, perhaps because turbulence usually intervenes before the 'Laminarast' is reached, but somewhat elaborate equations have been proposed in order to explain them (see Reiner, 1949).

It is of interest to establish whether such types of behaviour are characteristic of synovial effusions and, if so, under what conditions of flow.

EXPERIMENTAL

The fluids were tested in a viscometer (as originally described by Scott Blair (1941) for work on prenatal mammary secretions), consisting of a horizontal capillary tube attached at each end to relatively wide vertical tubes. Measurements were made in a thermostatically controlled water bath at 38°. The sample is sucked up into one of the side tubes and its rate of fall is then measured on a vertical scale. The initial height of the column above its rest position is $h_0 = 10$ cm. which represents half the head, since the column falls by an equal amount in the other arm of the viscometer. In a few experiments greater heads than this have been used but no extra information appears to be gained by this procedure, which increases the experimental errors. It was shown in the earlier paper (Scott Blair, 1941) that, for true fluids, the viscosity η, is given in terms of the time (t) taken for the head of the column to fall from h_0 to each observed value of h, by the equation

$$\eta = -\rho \frac{gR_c^4}{4LR_w^2} \frac{dt}{d(\ln h)}, \qquad (1)$$

when R_c and R_w are the radii of the capillary and the wide tubes, respectively, L is the length of the capillary, g is acceleration due to gravity, ρ is the density of the material which, for the present purposes, may be taken as unity.

Defining Δt as the time differences between successive (cm.) readings of h, this may be rewritten

$$\eta = -\rho \frac{gR_c^4}{4LR_w^2} \frac{\Delta t}{\Delta(\ln h)}, \qquad (1a)$$

or

$$\eta = -A \frac{\Delta t}{\Delta(\log h)},$$

where A is a constant for each capillary. (The minus sign means that head is falling.) For our standard viscometer, $A = 0{\cdot}000521$.

For a Newtonian fluid, since $\tau = h\rho g R_c/L$, and, by definition, $\dot{\gamma} = \tau/\eta$, we have

$$\dot{\gamma} = -\frac{4R_w^2}{R_c^3}\, h\, \frac{\Delta(\ln h)}{\Delta t}.$$

The side tubes are sufficiently wide for their resistance to be ignored.

Methods of expressing results in relation to experimental method

Provided that η is effectively constant, as was the case with the prenatal mammary secretions, equation (1) may be integrated (Scott Blair, 1941) to give:

$$\eta/\rho = \frac{t}{\ln(h_0/h)}\,\frac{gR_c^4}{4LR_w^2}. \qquad (2)$$

Curves for any single capillary may therefore be plotted as $t/\log(h_0/h)$ against some suitable function of h, or as t against $\log(h_0/h)$, as was done in the earlier paper using equation (2) which applies to a Newtonian fluid. When the anomalies are considerable, however, this is not a sound method, since, unless h_0 can be chosen to mark some well-defined, constant viscosity, such as the beginning of the 'Laminarast', its value is entirely arbitrary. Moreover, any function of h which is linear with $t/\log(h_0/h)$ for one arbitrary value of h_0 will not, in general, be strictly linear for other selected values, even though in practice the curvature may not be very noticeable.

Moreover, the use of the integrated form of the equation implies that the value of the 'viscosity' $(\tau/\dot{\gamma})$ for any given value of h is calculated for the range between the arbitrarily chosen h_0 and h and the actual values for 'viscosities' so calculated are therefore highly dependent on the choice of h_0.

For these reasons it is better to use the differentiated form of the equation (i.e. equation 1*a*) rather than equation (2), even though the use of the integrated form generally gives smoother curves having less scattered points.

Selection and preparation of samples

The samples of synovial fluid were all obtained from knee joints by aspiration with a wide-bore needle. The fluid was kept in a screw-capped bottle in a refrigerator. Shortly after aspiration most fluids form a fine clot. Before any measurements were made the fluid was centrifuged in order to separate this clot and any fibrin flakes or cellular debris which might obstruct the capillary.

The fluids tested were unselected, being from the knees of any patient in whom fluid was clinically demonstrable. Of the 63 samples tested, 34 were from 17 patients who suffered from rheumatoid arthritis, 13 samples from 4 patients with ankylosing spondylitis, 5 from osteoarthritis, 1 from Reiter's disease, and 10 from 3 patients with undiagnosed effusions.

RESULTS

The results of our experiments are best classified under two headings.

Anomalies of flow within a single capillary. For a Newtonian fluid, η is independent of τ and this is found to be nearly the case for the thinner synovial fluids (see lowest curve to Fig. 1). For thicker samples, however, this law does not hold.

Using a single standard capillary, we find that, for such samples, a plot of $\Delta t/\Delta(\log h)$ against $\log h$ gives excellent straight lines for effusions covering a very wide range of consistency.* The great majority of such curves drawn for more than sixty samples, show no sign of curvature. Among those that do show slight upward or downward curvature, of which there are about equal numbers, there is no relation observed between the direction of curvature and the consistency. Since for a single capillary, $h \propto \tau$, this plot may be expressed by the equation

$$\tau/\dot{\gamma} = \eta = \eta_0 + \alpha \log \tau, \qquad (3)$$

where α is the slope of the straight line. A few typical examples are shown in Fig. 1. The original data are given in Table 1.

A plot of $\log \dot{\gamma}$ in place of $\log \tau$ against η (where η is defined as $-A\Delta t/\Delta(\log h)$) also gives good straight lines for both sets of results. The equations represented by these two plots cannot both be strictly true. Apart from all else, if both were valid, then the values of $\log \dot{\gamma}$ should be linear with those of $\log \tau$ and this is not the case. Experimentally, we cannot decide which equation fits the data more closely, nor is there any obvious statistical procedure for doing so. Our preference for the $\log \tau$ plot is largely arbitrary.

Many biological fluids show some type of thixotropy (see Frey-Wyssling (1952)). Repeated shearing of the synovial fluids, however, leads to a slight increase in consistency. Thus, for example, the readings for a typical sample for the final value of $h = 2$ are shown in Table 2. On still further shearing a stage is reached at which the consistency does not change any further.

It was at first thought that this effect might be caused by some structure set up by the shearing which does not relax when the sample is left in the viscometer between the shears. However, when the sample was taken out of the viscometer and fairly thoroughly mixed before re-testing, a similar increase in consistency was found to have taken place. It does not seem likely that this change can have been due to evaporation and the mechanism of the process is obscure. Centrifuging for 20 min. instead of the usual 5 min. has no effect on the consistency.

Data from a few samples, when τ was plotted against $\dot{\gamma}$, show some tendency to inflect and to give a 'Laminarast', but this is only really clearly defined by the first two or three readings from the falling column. Using the differential form of the equation (equation 1*a*), the effect is much less marked than it is when the integrated form (equation 2) is used. This is to be expected, since in

* Consistency is defined as 'that property of a material by which it resists permanent change of shape' and is described by the complete flow-force relation.

Table 1. *Values of t (sec.) corresponding to readings of h (=half-head) for a number of synovial effusions* ($h_0 = 10$.)

h (cm.)		8	7	6	5	4	3	2
Sample no.	4	78	130	194	277	393	559	836
	25	27·0	44·9	68·7	98·7	141·5	203	304·8
	35	18·3	30·4	45·1	64·2	87·5	122·9	178·3
	39	35	58	88	127	180	260	388
	42	12	20	29	41	56	78	110
	44	7	11	17	23	31	41	57
	105	—	111	168	242	340	—	710

the former plot each point represents the viscosity of the sample covering a small region of stress (Δh) of which the plotted value of h forms the centre. In the latter plot, viscosity is interpreted over the whole range from h_0 to h; hence changes in viscosity are not so clearly shown.

Through the kindness of Dr A. G. Ogston and Dr J. E. Stanier, it has been possible to examine data obtained by them on six samples of bovine synovial fluid from the slaughter house, using a concentric cylinder viscometer. Except at the lowest rates of shear, where the points are somewhat irregular, equation (3) gives very satisfactory straight lines. However, the plots of two experiments on equine slaughter-house samples, tested by us in the capillary instrument showed marked curvature.

Effects of varying capillary radius and length. A number of rheologists, specifically Schofield & Scott Blair (1930) have shown that anomalies in flow in capillary tubes may be classified as of two kinds: (*a*) deviation from the Newtonian law that $\tau/\dot{\gamma} = \eta = \text{const.}$ although certain basic conditions are maintained. These are that each particle of the material flows in a straight line parallel to the wall of the tube, that there is a monotonic relation between τ and $\dot{\gamma}$ and that there is no slippage or sticking to the walls. (*b*) Conditions under which one or more of these three postulates do not hold.

Anomalies of type (*a*) lead to non-linear relations between τ and $\dot{\gamma}$; but, if data from different capillaries are plotted τ versus $\dot{\gamma}$, the curves should be unique. Schofield & Scott Blair, and later many others, have studied so-called 'sigma-effects' in which the curves are not unique and where the Poiseuille-Hagen fourth-power-radius law is not obeyed. Zamboni (1943) showed that mucins from many sources, as well as some unspecified synovial fluid, failed to obey the linear-length law, flow being relatively slowed down in longer tubes. Similar effects, both positive and negative, have been recorded for other materials (see Scott Blair, 1949).

Experiments using a number of capillaries differing in radius and length have shown that the fourth-power-radius law holds for our materials within the rather wide limits of experimental error. There is some evidence that the length law does not

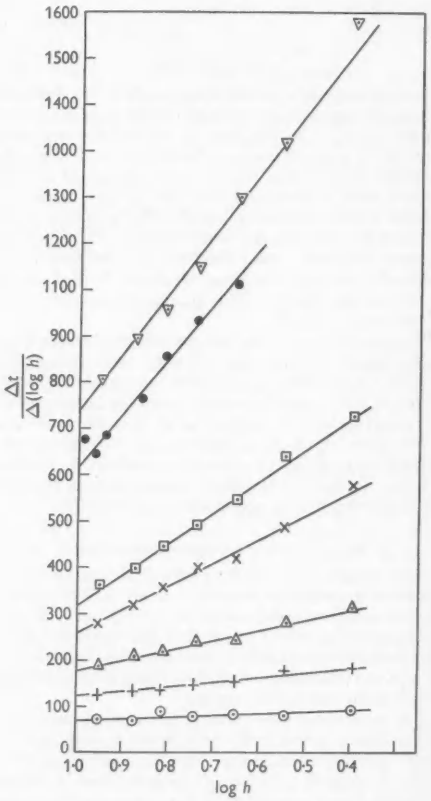

Fig. 1. Viscosity of synovial effusions (differential-definition) plotted against logarithm of stress (t in sec, h in cm.). The seven types of points refer to seven different samples: ▽, no. 4; ⊡, no. 39; ×, no. 25; △, no. 35; +, no. 42; ⊙, no. 44; ●, no. 105.

Table 2. *Effect of repeated shearing on consistency of a synovial effusion*

(t_2 is time taken for column to fall from $h_0 = 10$ to $h = 2$.)

No. of shear	1	2	3	4	5	6
t_2 (sec.)	285	291	292	295	297	299

hold perfectly, flow being relatively slower in the longer tubes. This effect, though marked only in the case of samples of high consistency, may well be similar in character to the flow-hardening phenomena reported by Zamboni (1943).

DISCUSSION

The use of the differential form of the equation (equation $1a$) eliminates the explicit arbitrary length h_0, but there still remains the problem of how to describe curves plotted in accordance with equation (3). Being straight lines, these curves require two parameters: the slope α, and a second parameter which we have defined as η_0, being the viscosity at an arbitrary stress corresponding to $h = 10$ cm.

From purely algebraic considerations, we should probably choose that value of $\log h$ (and hence h) corresponding to zero viscosity when the curve is extrapolated. But it is clear that equation (3) will certainly not hold to this limiting condition, since the viscosity will never fall below that of water.

Thus, for want of any well-marked stress or shear rate clearly characterizing the material, the arbitrary choice of $h = 10$ cm. for a point at which to quote viscosity measured in this way can hardly be improved upon.

The results given in the last section show that, except in the case of effusions of very high consistency, the effects of radius, thixotropy and even length can be ignored for many purposes. Each sample may thus be designated by the two constants from equation (3). Of these two properties, η_0 represents the viscosity at comparatively high shear rates and α, which has also the dimensions of a viscosity ($ml^{-1}t^{-1}$), defines the rate at which viscosity changes with changing stress, i.e. the viscous anomaly. It is not surprising that the magnitude of the viscous anomaly should be a function of η_0, especially since viscosity is itself defined as $\tau/\dot{\gamma}$; but it is, perhaps, remarkable how close is the relationship. Fig. 2 shows a scatter curve for fifty-four samples. The correlation coefficient is 0·958. It is seen that, statistically, the anomaly only appears when η has reached a certain well-defined value. Using the standard viscometer, $\eta_0^* = 50$ corresponds to $\eta_0 = 0{\cdot}052 \times 50 = 2{\cdot}60$ centipoise. (Starred symbols represent values for a single capillary, not converted into absolute units.)

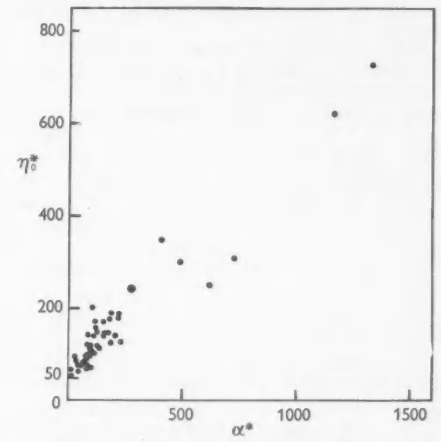

Fig. 2. Scatter diagram relating viscosity at high stress to viscous anomaly. (Rise in viscosity with falling stress.) η_0^* and α^* in arbitrary units.

Below this viscosity, given by the extrapolated intercept of the scatter curve, samples are, to all intents and purposes, Newtonian fluids, since $\alpha = 0$. For higher viscosities, and presumably when the concentration of mucin reaches a value large enough to produce marked intermicellar interference, there is a clear linear (statistical) relation between η_0 and α, the anomaly increasing progressively with increasing viscosity. This does not mean, however, that the individual samples can be described by a single parameter. Thus the ratio of $\eta_0^* - 50$ to α^* varies quite widely between individual samples, corresponding to their individual positions on the scatter curve. The extreme values are 1·48 and 0·25. This ratio may even eventually prove to be of clinical significance. Even with its two parameters, however, equation (3) represents a very simple law of flow for such complex materials which, as far as we are aware, has not been used previously by rheologists for any material.

Clinical value of results. Previous observations on the viscosity of pathological synovial fluids have taken no account of its non-Newtonian properties. As a consequence, normal values in various diseases have been expressed in terms of relative viscosity alone. Ropes, Robertson, Rossmeisl, Peabody & Bauer (1947) give the relative viscosities of synovial fluid for a number of diseases. We are now able to give true figures for the stress anomaly (α) and a viscosity (η_0) at a chosen stress. This gives a more complete picture of the behaviour of these fluids.

Since the samples were mostly from cases of rheumatoid arthritis, an idea of the range of viscosity in this condition is obtained. These are all fairly low, ranging from 2·9 to 10·3 centipoise.

That these results are not of specific diagnostic importance is shown, however, by the similarity of viscosities and stress anomalies shown by fluids from other conditions. Many more readings will have to be made before these results can be compared with the ranges given by Ropes *et al.* (1947).

An interesting observation is that when a joint is repeatedly emptied, neither the viscosity nor the

quantity of effusion remains constant. It appears that the larger the amount of the effusion from any given joint, the lower is the viscosity. The emptying of the joint thus alters both the quantities and the quality of the effusion. These findings show that in any *in vivo* experiment, account must first be taken of the variations resulting from the process of aspiration itself.

However, taking the whole group of patients with rheumatoid arthritis on whom these studies have been made, without repeated aspiration, no correlation could be found between the size of the effusion and its viscosity; nor did the acuteness of the effusion, the erythrocyte sedimentation rate, sex, age, nor duration of the disease show any relationship to the viscosity.

The importance of these rheological properties of synovial fluid is likely to be more in their relationship to the biochemical changes occurring in the formation of effusions than as direct clinical diagnostic tests.

SUMMARY

1. An examination of over sixty samples of human synovial effusions in a capillary viscometer has demonstrated relatively simple rheological behaviour. While thinner samples are almost true fluids, for the thicker samples, especially at high stresses, an anomalous viscosity appears. The anomalous viscosity may be described in terms of a parameter also having the dimensions of viscosity, equal increments of which correspond to progressive halvings of the remaining stress.

2. Although individual samples thus require two parameters for their specification, these are very highly correlated statistically. Anomalous viscosity starts when a certain basic viscosity ($\sim 2{\cdot}6$ centipoise) is reached and is thereafter closely proportional to the normal viscosity term.

3. Synovial fluids show no serious divergence from the Poiseuille-Hagen fourth-power law in relation to capillary radius and only a slight increase in viscosity with increasing capillary length, except in the case of effusions of very high consistency, which seem to flow less readily in the longer tubes. Repeated shearing or stirring causes a small but definite increase in viscosity, the cause of which is not known.

4. Since no obvious relation has been found between the viscosities, normal and anomalous, and acuteness of effusion, erythrocyte sedimentation rate, sex, age, or duration of disease, it is probable that their importance will be found to lie more in their connexion with biochemical changes occurring during the formation of the effusions, than in their use as diagnostic criteria.

We are indebted to Mr C. P. Cox for helpful discussions and useful suggestions, and Dr H. A. B. Simons for technical advice. Prof. J. McCunn of the Royal Veterinary College provided us with samples of equine synovial fluid. Facilities and assistance were made available by the Endowment Fund Subcommittee and the Board of Governors of The Royal Free Hospital.

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Some Factors Influencing the Orientation of ε-Amino Groups in Monolayers of Proteins and Amino Acid Polymers

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Interest has recently been focused on the specificity of the order of the amino acid residues along the polypeptide chains of proteins (Sanger, 1952). This in turn raises the further question, how does the amino-acid order affect the properties of the protein? In this paper we shall discuss the relative orientations of the ε —NH_2 group of lysine in monolayers spread at both the air-water and oil-water interfaces. In the various proteins and amino acid polymers studied there is evidence that these orientations depend on the nature of the neighbouring amino acid residues, on the constitution of the non-aqueous phase, and on the film pressure and the pH.

The ε —NH_2 groups are of importance in determining biological activity. Recent comprehensive reviews by Fraenkel-Conrat & Fraenkel-Conrat (1950) and by Olcott & Fraenkel-Conrat (1947) may be referred to in this connexion. Further, Reid (1951) found that the activity of certain growth-promoting hormones depended on the integrity of these groups, although the terminal α-amino groups were unimportant. Porter (1948) observed that the number of ε —NH_2 groups available for chemical reaction under slightly alkaline conditions depended on whether or not the protein was denatured. In particular, native β-lactoglobulin and horse-serum pseudoglobulin gave the reaction characteristic of free ε —NH_2 for only 19/31 and 57/80 of the groups. Only after denaturation in various ways were practically all the groups available.

Evidence of the effects of interfaces on protein activity has also been presented. A general discussion is given by Haurowitz (1950). For example, haemin is a very active lipoxidase in emulsions, but not in homogeneous solutions, according to Haurowitz & Schwerin (1940). Holtfreter (1946), studying the detailed development of amphibian eggs, found that interaction must occur between the protein yolk-platelets and the lipochondria (lipids enclosed in a protein envelope) before normal development can proceed. The surfaces of the lipochondria, he suggested, contain proteolytic enzymes which are inactive till the lipochondria and the platelets meet. The former, in the ensuing spreading across the surface of the platelets, cause these proteolytic enzymes in the surface to take up an activated configuration: the yolk becomes disintegrated, fat droplets are liberated (by rupture of the protein envelope) and the embryo develops. Needham (1951) has suggested that the interpretation of this sudden enzymic activity lies in changes in the configurations of the protein molecules at different interfaces.

Here we shall discuss from a physico-chemical point of view the specific interfacial effects, both on the ε —NH_2 groups of the lysine side chains and on the other amino acid residues in the peptide chain.

EXPERIMENTAL

The method of measuring interfacial potentials of monolayers has already been described (Davies, 1951*a*). Horse haemoglobin was kindly provided by Dr D. F. Cheesman; the bovine-serum albumin and γ-globulin were the crystalline products of Armour and Co.; gliadin was kindly supplied after careful purification, by Western Regional Laboratory, California, and the insulin was the product of Boots Pure Drug Co.

Of the synthetic amino acid polymers spread as possible model systems for the proteins, poly-L-lysine (mol.wt. probably of order of 6000) was kindly provided by Dr R. S. Hannan, Cambridge; poly-DL-leucine (degree of polymerization, 200) and the 1:1 co-polymer mol.wt. 1450) of γ-methyl-L-glutamic acid with DL-phenylalanine through Courtaulds Ltd., Maidenhead. The 1:1 co-polymer of DL-lysine with L-glutamic acid (mol.wt. 5700) and the 1:1:2 co-polymer of L-lysine, L-glutamic acid and L-leucine (mol.wt. 35000) were both provided through the courtesy of Dr H. Tani, Osaka University, Nakanoshima, Japan.

Except for the gliadin, the protein monolayers were spread from 0·1% solution in a 60% (v/v) solution of propanol in water, 0·5 M with respect to sodium acetate. This technique is that of Ställberg & Teorell (1939). This solution gives excellent spreading at both the oil-water and the air-water interfaces, even when the spreading is occurring against the pressure of the film already in the surface (Alexander & Teorell, 1939). Gliadin was spread from 60% (v/v) ethanol-water solution, 0·1% with respect to gliadin.

The polyleucine and the co-polymer of γ-methylglutamic acid with phenylalanine spread easily and rapidly from 0·1% solution in anhydrous dichloroacetic acid to which about 10% propanol was added to assist film formation. The poly-lysine, the poly-lysine-γ-methylglutamic acid and the 1:1:2 co-polymer of lysine, glutamic acid and leucine

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were all soluble (0·1%) in a solution consisting of 60% water and 40% (v/v) propanol. From this solution spreading was immediate.

All reagents were A.R. grade, used without further purification. Solvents, including water, were distilled in an all-glass Pyrex apparatus. The oil phase consisted of redistilled A.R. light petroleum, b.p. above 120°. All experiments were carried out at room temperature, 20±2°. Unless otherwise stated, substrates at pH 2, 6·8 and 13 consisted of 0·01 N-HCl, 0·02 M phosphate buffer and 0·1 N-NaOH, respectively.

RESULTS

In Fig. 1 the results are expressed as the difference between the air-water (A/W) and oil-water (O/W) potentials at an area of 1·6 m.² per milligram. It may be noted that at acid and neutral pH's ΔV (A/W) > ΔV (O/W), holds for all the proteins studied and for all the synthetic amino acid polymers. On alkaline solutions the difference is much less: indeed, for films of haemoglobin, insulin and serum albumin, there is a reversal of sign at higher areas per milligram, and ΔV (A/W) < ΔV (O/W). It is upon this reversal, which decreases as the film is compressed, that attention has been concentrated. Among the amino-acid polymers no reversal is shown except by the co-polymer of lysine, glutamic acid and leucine. This will be discussed more fully below.

From the potentials ΔV, the mean apparent surface dipole moments, μ, per amino acid residue can be calculated. For the amino acid polymers this is possible precisely using the known residue molecular weights; for the proteins a mean area of 20 Å² per residue is assumed, leading to the μ values in Fig. 2 (for serum albumin). The slopes of the μ/A plots are shown in Table 1. We define μ by use of the Helmholtz equation, $\Delta V = 4\pi n\mu$, for both neutral and electrically charged films: it therefore includes any contributions from the underlying diffuse ionic double layer (Davies, 1951*b*). In the formula, n is the number of residues on each square centimetre of the interface.

DISCUSSION

Branching and cross-linking in the peptide chain. This does not seem to be an important factor in the spreading of proteins, in that they form unimolecular films so readily. Further, all the effects shown by protein films at the O/W and A/W interfaces at pH's 2, 6·8 and 13 can be reproduced with suitable films of amino acid polymers, the molecules in which are unbranched. We may conclude that cross-linking and chain branching are not important under these conditions. Although the structure of the proteins may be affected by the extreme pH limits, these were chosen to ensure complete ionization of the protein molecules.

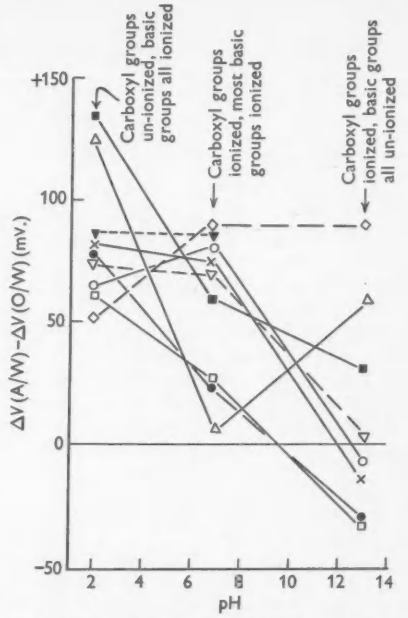


Fig. 1. Variation of (ΔV (A/W) − ΔV (O/W) at 1·6 m.² mg.⁻¹ at the pH's 2, 6·8 and 13. The reversal at pH 13 is attributed to a reorientation of the ϵ —NH_2 groups when these are un-ionized. ◇, Poly-1:1-γ-methyl-L-glutamyl-DL-phenylalanine; ▼, poly-lysine; △, poly-1:1-DL-lysyl-glutamic acid; ■, polyleucine; ▽, gliadin; ○, insulin; ×, 1:1:2 co-polymer; ●, haemoglobin; □, serum albumin.

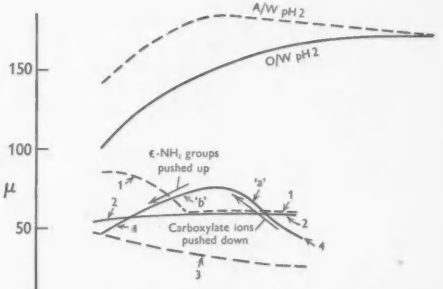


Fig. 2. Surface dipole moments, μ (in milli-debyes per amino acid residue), calculated from the Helmholtz equation $\Delta V = 4\pi n\mu$ for serum albumin. Curve 1, pH 6·8, A/W; curve 2, pH 6·8, O/W; curve 3, pH 13, A/W; curve 4, pH 13, O/W. The dipole moments reflect both the electrical charge on the film and the orientations of such polar groups as —NH_2.

Table 1. *Slopes of the μ/A plots for monolayers at* pH 13

(The units are milli-debyes per square metre. A positive sign means that μ decreases as the film is compressed and vice versa.)

Substance	Wt. of lysine/100 g. of protein or polymer*	Area of film per milligram (m.²) 3·4	2·4	1·4
Serum albumin	13 (12·4)	A/W 0 O/W −31	A/W −9·6 O/W 0 (max.)	A/W −9·6 O/W +26
Haemoglobin (horse)	8 (8·6)	A/W 0 O/W −6	A/W 0 O/W 0 (max.)	A/W −22 O/W +30
Insulin	1·3 (2·5)	A/W 0 O/W 0	A/W −6 O/W −6	A/W −6 O/W 0
Gliadin	0·7	—	A/W −12 O/W 0	A/W +30 O/W +30
1:1:2 co-polymer of glutamic acid with lysine and leucine	22 (calc.)	A/W 0 O/W −6	A/W −10 O/W 0 (max.)	A/W −12 O/W +28
Co-polymer of γ-methylglutamic acid and phenylalanine (+2N-NaCl)	0 (calc.)	A/W — O/W −20	A/W — O/W −21	A/W — O/W −30

* The first four values are those quoted by Cohn & Edsall (1943), the figures in brackets are means of those quoted by Tristram (1949).

Amino acid composition. At pH 2 all the carboxyl groups will be un-ionized, and all the basic groups will be fully ionized. The latter will raise the surface potential, ΔV. There is, however, no correlation between ΔV and the numbers of free cationic groups, suggesting that specific steric factors may alter the orientations of the permanent dipoles (the —CO.NH— groups) and so destroy the expected agreement. Further, the ionized side chains are likely to be pulled down into the aqueous phase to a position so far below the water surface that counter-ions can nearly surround the charged groups, lessening their contribution to ΔV.

Orientation of the side chains. Not only does the lack of correlation between amino acid composition and ΔV suggest that there are important steric effects influencing the surface potentials, but the curious reversal in the sign of

$$(\Delta V\,(\mathrm{A/W}) - \Delta V\,(\mathrm{O/W}))$$

for the proteins at pH 13 (except gliadin) leads to the same conclusion. As shown in Fig. 1, this is greatest for serum albumin, which also has the highest lysine content of the proteins studied.

The relationship between $(\Delta V\,(\mathrm{A/W}) - \Delta V\,(\mathrm{O/W})$ at pH 13 and the number of grams of lysine per 100 g. protein is shown in Fig. 3. While the results for the proteins lie on a smooth curve, those for the amino acids polymers do not, suggesting that it is the immediate environment of the lysine groups which is important. We shall return to this point later. No correlation with either glutamic acid or glutamine contents is found.

That the values of $(\Delta V\,(\mathrm{A/W}) - \Delta V\,(\mathrm{O/W}))$ at pH 13 are related to the lysine content shows the importance of the orientation of the chains bearing the ϵ —NH_2 groups. At this pH these groups will be

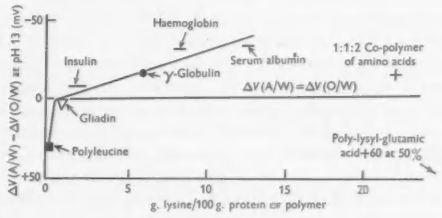
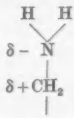


Fig. 3. ΔV (A/W) − ΔV (O/W) at pH 13 is plotted against wt. % lysine residues (Table 1).

completely un-ionized, and, because of the rather long hydrocarbon chain —$(CH_2)_4$—, there will be three possible positions for the side chains at the oil-water interface: in the oil, in the water, and flat in the interface.

The μ/A plots enable us to deduce which orientation actually occurs. At the O/W interface the μ/A curve falls steeply below about 2·2 m.² mg.$^{-1}$ at pH 13 as in Fig. 2 and Table 1. This suggests that here, in the compressed film, the ϵ —NH_2 groups are being pushed up into the oil, the dipoles (due to the small charges $\delta+$ and $\delta-$) being arranged to an increasing extent in the following manner:



The contribution of these will be negative upwards, so that the total μ falls sharply. Similar effects have been found for chains carrying an ω —Br group (Davies, 1953). The rather flat maximum in μ at the O/W interface at pH 13 for serum albumin,

haemoglobin and the 1:1:2 amino acid polymer lies between 2·1 and 2·5 m.^{2} mg.$^{-1}$. This area per milligram is the limiting area of the peptide 'backbone' (i.e. 1·0 m.^{2} mg.$^{-1}$) plus the area of a lysine side chain, $-(CH_2)_4NH_2$, which is calculated from models to occupy an area of 1·2 m.^{2} mg.$^{-1}$ when lying flat in the interface. Therefore, below, an area of 2·2 m.^{2} per milligram it is to be expected that the lysine side chains will be expelled from the interface; the sharp decrease in μ tells us that they are pushed upwards into the oil. The energy to accomplish this would be prohibitively high for an ionized side chain.

The rise of μ as the low-pressure O/W film is compressed at pH 13 (region '*a*', Fig. 2) reflects the tendency for the glutamic side-chains to be pushed below the aqueous surface. Since these bear a negative electrical charge, the work required to force them up into the oil would be prohibitively great. Below the aqueous surface their effect (negatively) is less, and so μ rises markedly on compression (slope negative). As an example, the co-polymer of γ-methylglutamic acid and DL-phenylalanine shows (Table 1) a pronounced electrical effect of this type at pH 13. We may also note that there is an approximate correlation between the magnitude of this effect (the negative slope at the O/W interface at pH 13) and the content of free glutamic acid plus aspartic acid plus the free carboxyl end group. Glutamine (as in gliadin) is not ionized, and is not included. The data for the content of the free acids are available in reviews by Olcott & Fraenkel-Conrat (1947) and by Tristram (1949).

At the A/W interface at pH 13, on the other hand, μ remains nearly constant at high areas, although on further compression of the monolayer there is (except for gliadin) a slight increase of varying magnitude ($d\mu/dA$ is negative). This may be explained as follows: if the lysine residues find themselves in an environment consisting largely of cohering hydrocarbon chains packed vertically, they will penetrate into them, also becoming oriented vertically. This is shown diagrammatically in Fig. 4. A rather similar effect has been reported for long-chain ω —Br acids (Gerovich & Frumkin, 1936). At the O/W interface the film must be highly compressed before the ϵ $-NH_2$ groups pack in this manner, and until this happens

$$\Delta V\,(\mathrm{A/W}) < \Delta V\,(\mathrm{O/W}).$$

Order of the amino acids in the peptide chain. The only protein of those studied here in which the arrangements of the amino acids along the chain has been determined is insulin (Sanger, 1952). In this substance the order of the residues about the lysine groups is probably as follows:

Glu.Arg.Gly.Phe.Phe.Tyr.Thr.Pro.Lys.Ala
(C-terminal group).

Although the chains will, even when all the carboxyl groups are ionized, contain folds both in solution and at the A/W surface, it is clear that in the environment of the lysine group there is a relatively large concentration of bulky, hydrocarbon groups. A simple calculation shows that in the last seven residues (including the lysine group) there are 60% of such large groups (phenylalanine, tyrosine, proline, leucine), as against the average of 30% along the whole chain. The effect of neighbouring chains or folds on the orientation of the ϵ $-NH_2$ group will also be important, and will make difficult or impossible any interpretation in terms only of the types of neighbours in the same peptide chain. Nevertheless, we may predict from the fact that $(\Delta V\,(\mathrm{A/W}) - \Delta V\,(\mathrm{O/W}))$ is negative for serum albumin and for haemoglobin at pH 13 (Fig. 3) that the lysine side chains will be largely surrounded by large hydrocarbon groups which will cohere strongly and pack vertically at the A/W interface.

Some common feature in protein organization seems likely from the continuity of the curve in Fig. 3 for the different proteins. That the co-polymer of lysine, leucine and glutamic acid does not fall on the same curve as the proteins suggests that the (probably statistical) arrangement of its lysyl, glutamic and leucyl residues is not conducive to a large reversal of the order of the potentials. In the latter material there will often be several glutamate ions held near the lysine; under such conditions a sufficiently strong cohesion to lift the lysine groups vertically will not easily be attained on account of the electrostatic repulsion. This is shown even more clearly for the co-polymer of lysine with glutamic acid, no negative value for $(\Delta V\,(\mathrm{A/W}) - \Delta V\,(\mathrm{O/W}))$ being found at pH 13 owing to the excessive density of negative charge and the absence of large hydrophobic residues.

That lysine alone among the amino acids should show these specific effects is not difficult to understand. The long hydrocarbon chain $-(CH_2)_4-$ is partly responsible; glutamic acid, with its shorter $-(CH_2)_2-$ chain does not show the same effects at pH 2. Arginine and histidine, also un-ionized at pH 13, have much higher ratios of polar to non-polar groups in the residue, which in consequence will be lifted above the surface only with much greater difficulty.

If the ϵ $-NH_2$ group is ionized (its pK is 9·4–10·6) the free energy of either placing this ion in the non-aqueous phase or of removing the proton

$$-NH_3^+ \rightarrow -NH_2 + H^+$$

will be far higher than the free energies available in adsorption and reorientation. Only the un-ionized polar group of lysine, attached to a long hydrocarbon chain, will readily become 'unavailable' to

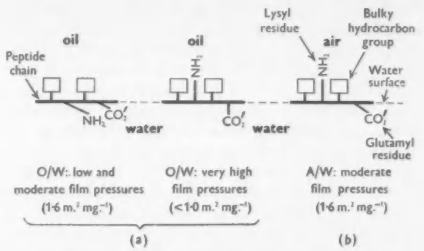


Fig. 4. Orientations of the $-(CH_2)_4NH_2$ chains in proteins at (*a*) the oil-water interface, and (*b*) the air-water interface.

the aqueous phase either in A/W monolayers at moderate pressures or in the native protein (where the van der Waals forces of cohesion between the hydrocarbon side chains are also very powerful). When the protein is unfolded at a lipid-water interface the ϵ—NH_2 is much more readily available (Fig. 4), and in this we may find some explanation of the curious and specific biological phenomena mentioned in the introduction.

SUMMARY

1. The potentials of monolayers of various proteins and amino acid polymers, including a new synthetic amphoteric amino acid polymer, have been measured at the interfaces air-water and oil-water, at pH's 2, 6·8 and 13. With this new polymer (poly-1:1:2:-L-lysyl-L-glutamyl-L-leucine) all the phenomena shown by protein films can now be reproduced. Other polymers studied were poly-L-lysine, poly-DL-leucine, poly-1:1-γ-methyl-L-glutamyl-DL-phenylalanine and poly-1:1-DL-lysyl-L-glutamic acid.

2. At pH 13 the un-ionized ϵ—NH_2 groups of the lysine residues tend to enter the non-aqueous phase, especially at the air-water interface when the neighbouring amino acid residues in the peptide chain are favourable.

3. The 'unavailability' of such ϵ—NH_2 groups at this interface and in the native protein may be of major biological importance.

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A Colorimetric Method for the Determination of the Principal Metabolites of Nicotinic Acid in Human Urine

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Recent work has shown that *N*-methyl-2-pyridone-5-carbonamide and *N*-methylnicotinamide are the main metabolites of nicotinic acid normally excreted in human urine (Holman & Lange, 1949; 1950), that no more than a trace of nicotinamide is excreted (Reddi & Kodicek, 1953), and that nicotinic acid, *N*-methylnicotinuric acid betaine and pyridine nucleotides, and probably nicotinuric acid, are not normally present (Reddi & Kodicek, 1953). It therefore seems likely that the sum of the urinary excretions of *N*-methyl-2-pyridone-5-carbonamide and *N*-methylnicotinamide, expressed as nicotinic acid, may prove to be a reliable index of nicotinic acid status in man. Existing methods for the determination of these metabolites, however, are laborious and time consuming and, since the

methods involve quite different techniques, it is difficult for one analyst to determine both substances at the same time.

It has also been shown (Holman & Lous, 1951) that *N*-methyl-2-pyridone-5-carbonamide tends to distribute itself in the total body water of man. It is possible that, if a simple method were available for its estimation, this pyridone might provide the basis for a method of estimating body water.

The present investigation was made in an attempt to devise a rapid colorimetric method for the simultaneous determination of *N*-methyl-2-pyridone-5-carbonamide and *N*-methylnicotinamide, based on the conversion of the carbonamide group into an amino group by treatment with hypobromite, and diazotization and coupling of the amino compound formed to yield an azo colour. The hypobromite reaction (Hofmann reaction) has long been used in preparative chemistry, but was not thought to have been adapted for analytical purposes. Although there was a possibility that urine might contain substances capable of giving azo colours by the diazotization process alone, it seemed unlikely that any urinary constituent other than derivatives of nicotinic acid would require both hypobromite treatment and diazotization for the development of an azo colour, since this series of reactions is specific for aromatic and heterocyclic amides.

After the present work had been completed, it was discovered that Goodyear & Murphy (1944) had proposed a method based on the Hofmann reaction for the estimation of nicotinamide in pharmaceutical preparations. They did not, however, apply their method to other metabolites of nicotinic acid nor to the analysis of biological materials. The relatively large amount (75–325 μg.) of nicotinamide required for a determination was an important factor limiting the application of the method.

EXPERIMENTAL

Preliminary experiments showed that a purple azo colour could be formed from *N*-methyl-2-pyridone-5-carbonamide by heating with hypobromite solution, and then acidifying the solution and diazotizing and coupling according to the technique of Bratton & Marshall (1939), as modified by Smith, Finkelstein, Aliminosa, Crawford & Graber (1945). A colour was not always formed, however, particularly when the amount of hypobromite was large in relation to that of the pyridone. The amount of bromine utilized during the reaction with the pyridone, as estimated by thiosulphate titration, increased with the excess of hypobromite added up to as much as 8 g. moles Br/mole of pyridone. Since only 0·5 g. mole Br/mole is required for the Hofmann reaction (e.g. see Sidgwick, Taylor & Baker, 1937), these results suggested that side reactions occurred, probably leading to extensive oxidative breakdown of the pyridone molecule.

Side reactions could probably have been largely prevented by avoiding an excess of bromine, but this technique is not likely to be suitable for analytical purposes, particularly in the presence of urine, which contains many substances capable of reacting with hypobromite. An alternative possibility was examined, namely, adding an excess of hypobromite at room temperature to ensure combination with the carbonamide group of the pyridone, and then removing the excess before heating the solution. Of various substances tested as agents for the removal of excess of bromine, phenol was the most satisfactory, enabling intense colours to be obtained even when a large excess of hypobromite had previously been added to the pyridone. Under suitable conditions a colour could be formed in the presence of as much as 5000 g. moles Br/mole pyridone. By the use of phenol, azo colours could also be produced from nicotinamide, *N*-methylnicotinamide and *N*-methyl-2-pyridone-3-carbonamide. In every case the amount of test substance required to give a colour of suitable intensity for colorimetric measurement was 1–50 μg. The chief optical properties of the colours formed are summarized in Table 1.

A trace of brownish yellow colour was produced by the reagents in the absence of metabolites of nicotinic acid. This colour increased in intensity during the first 45 min. after addition of the coupling agent, but altered only slightly thereafter. Its absorption at 590 mμ. was equivalent to about 0·4 μg. of *N*-methyl-2-pyridone-5-carbonamide, and that at 500 mμ. to about 3 μg. of *N*-methylnicotinamide chloride. Coenzyme I (diphosphopyridine nucleotide) did not yield a colour, the trace of orange produced by a preparation of this substance being accounted for by the nicotinamide which was present as an impurity.

Optimum conditions of heating and concentrations of NaOH, bromine and phenol were ascertained. Nicotinamide and *N*-methylnicotinamide required for maximal colour production a slightly more alkaline hypobromite solution than the pyridones. It was also found with each of

Table 1. *Properties of azo colours produced*

Substance	Colour produced	Time to reach maximal intensity	Light absorption at various wavelengths*			
			500 mμ.	570 mμ.	580 mμ.	590 mμ.
Nicotinamide	Orange	8 min. (after 30 min. fades 4%/hr.)	100	—	1·2	0·5
N-methylnicotinamide	Orange	8 min. (after 30 min. fades 4%/hr.)	100	—	2·7	0·3
N-methyl-2-pyridone-5-carbonamide	Purple	1 hr. (stable 1–6 hr. then fades 0·1%/hr.)	45	100	98	92·5
N-methyl-2-pyridone-3-carbonamide	Blue	2·5 hr. (stable 2·5–21 hr.)	27	98	100	98

* As percentage of maximal value.

the four substances that colour production could be considerably increased by protecting the solution from light during the reaction. On the basis of these considerations a method was worked out which was suitable for the colorimetric determination of any one of the four substances when present alone in pure solution.

Recommended method for colour development

The following solutions are required:

Bromine solution. 12·5 g. NaBr and 12·5 g. bromine (4 ml.) were dissolved in water and diluted to 100 ml. The solution was stable in the refrigerator for at least 2·5 months.

Sodium hypobromite solutions. (*a*) Pyridone reagent. 4 ml. 2·5 N-NaOH were diluted with water to about 45 ml., 2 ml. bromine solution added, and the solution was made to 50 ml. with water. The solution was stable for not more than 24 hr. in the dark. (*b*) Reagent for *N*-methylnicotinamide and nicotinamide. Instead of 4 ml., 6 ml. of 2·5 N-NaOH were used.

Other reagents. Aqueous phenol (0·45%, w/v; stable in refrigerator for at least 2 weeks); 2·4 N-HCl; $NaNO_2$ (0·1%, w/v; stable in a stoppered bottle in the dark for 3 days); ammonium sulphamate (0·5%, w/v; stable in a stoppered bottle in the dark for 2 weeks); *N*-(1-naphthyl)-ethylenediamine dihydrochloride (0·1%, w/v) (stable in refrigerator for at least 3 months).

Standard solutions. These were solutions of *N*-methyl-2-pyridone-5-carbonamide, *N*-methyl-2-pyridone-3-carbonamide, nicotinamide and *N*-methylnicotinamide chloride, containing 50 μg./ml.; the *N*-methylnicotinamide solution was preserved by making 0·1 N with HCl, the remaining solutions by addition of a little $CHCl_3$; all solutions were stored in a refrigerator and were stable for at least a month.

Procedure. A measured volume of an aqueous solution at pH 7–9 containing 0–50 μg. of nicotinamide, *N*-methylnicotinamide, *N*-methyl-2-pyridone-5-carbonamide, or *N*-methyl-2-pyridone-3-carbonamide, was transferred to a Pyrex, rimmed, 6 × $\frac{3}{4}$ in. test tube and diluted to 4 ml. with water. Light being excluded, 1 ml. sodium hypobromite solution was added and 4 min. later 1 ml. phenol solution. A stopper was inserted and held in place by means of a metal cap (Fig. 1), and the tube heated for 10 min. in a vigorously boiling water bath. After being cooled in running tap-water for 4 min., the cap was removed and 0·5 ml. 2·4 N-HCl was added, followed by 0·5 ml. $NaNO_2$ solution. Four min. later 0·5 ml. ammonium sulphamate solution was added, and again 4 min. later 0·5 ml. of *N*-(1-naphthyl)-ethylenediamine dihydrochloride solution. The stopper was inserted and the contents of the tube were well shaken immediately after each addition. A stop-watch was used to time the intervals between additions. Up to twelve tubes could be carried through this procedure at the same time.

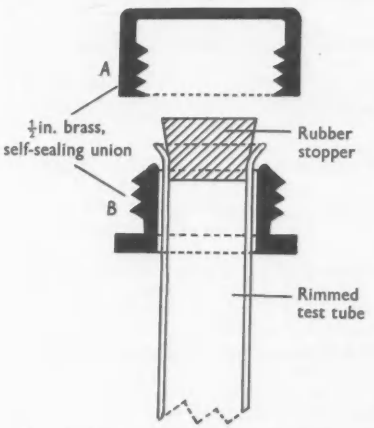

Fig. 1. Method of sealing test tubes during heating process. The bore of part *B* of the union was sufficiently enlarged to allow the test tube to be passed through as far as the rim. The stopper was inserted in the tube and parts *A* and *B* screwed together.

Colour measurement. The colour intensity was measured against a reagent blank prepared at the same time, using a spectrophotometer with 1 cm. cells at a wavelength of 500 mμ. for nicotinamide and *N*-methylnicotinamide and at 570–590 mμ. for the pyridones. A Spekker photoelectric absorptiometer with Spectrum Blue-green (480–500 mμ.), or Spectrum Yellow (570–590 mμ.), filters could also be used. Readings for nicotinamide and *N*-methylnicotinamide were taken at least 10 min. after addition of the coupling agent, readings for *N*-methyl-2-pyridone-5-carbonamide after at least 1 hr. and those for *N*-methyl-2-pyridone-3-carbonamide after at least 2·5 hr.

Calculation of results. The amount of the test substance present was obtained by reference to a calibration curve prepared at the same time with known amounts of the same substance.

Application of the method to urine

Analysis of mixtures. Since the colours given by *N*-methylnicotinamide and nicotinamide showed no appreciable absorption at 570–590 mμ. (see Table 1), neither of these substances interfered seriously with the determination of *N*-methyl-2-pyridone-5-carbonamide or *N*-methyl-2-pyridone-3-carbonamide. The error was reduced to a minimum by reading at 590 mμ. Mixtures of the two pyridones could be analysed but the individual pyridones could not be differentiated, and neither *N*-methylnicotinamide nor nicotinamide could be determined in the presence of other colour-producing substances. In the analysis of urine, *N*-methyl-2-pyridone-5-carbonamide could be determined in the presence of all other metabolites of nicotinic acid, since *N*-methyl-2-pyridone-3-carbonamide is not excreted (Holman & Lange, 1949). *N*-Methylnicotinamide could be determined only after separation from *N*-methyl-2-pyridone-5-carbonamide and nicotinamide.

Separation of N-methylnicotinamide from N-methyl-2-pyridone-5-carbonamide and nicotinamide. Since *N*-methylnicotinamide is a relatively strong base compared with the latter two compounds, attempts were made to effect a separation by differential adsorption on Decalso. Under the conditions of the method of Hochberg, Melnick & Oser (1945) for the adsorption of *N*-methylnicotinamide from urine, *N*-methyl-2-pyridone-5-carbonamide was not adsorbed to a significant extent, but partial adsorption of nicotinamide occurred. By raising the pH of adsorption from 4·5 to 7, however, it was possible to prevent completely the adsorption of nicotinamide and *N*-methyl-2-pyridone-5-carbonamide without interfering with the adsorption of

N-methylnicotinamide. After washing of the Decalso, the adsorbed *N*-methylnicotinamide was eluted with 25% KCl solution and determined colorimetrically in the absence of the other two metabolites. In a typical experiment the recovery of *N*-methylnicotinamide in the KCl eluate was 96% in the presence, and 88% in the absence, of urine.

Methods for removal of interfering substances from urine. When the colour reaction was carried out in the presence of urine, colour production was to some extent inhibited (see data for untreated urine in Table 2). Interference increased with the volume of urine added and also with the concentration of the particular specimen used. In order to render more comparable the results obtained with urines of differing degrees of concentration, samples of urine were measured in terms of the volume of urine excreted in a given time (see Table 2).

In the separation of *N*-methylnicotinamide by adsorption on Decalso, most of the interfering substances in urine passed through in the filtrate and washings. Samples of the KCl eluate representing up to 0·8 min. excretion of urine could be analysed directly for *N*-methylnicotinamide without inhibition of colour development. Untreated urine, however, could not be analysed directly.

A partial removal of interfering substances from urine was effected, without altering the concentrations of nicotinic acid metabolites present, by shaking with $Pb(OH)_2$. After this treatment samples of up to 0·1 min. excretion could be analysed for pyridone without appreciable inhibition of colour development (see Table 2). A more complete purification was obtained by adsorbing the *N*-methyl-2-pyridone-5-carbonamide in urine on Lloyd's reagent, eluting with NaOH solution, and then shaking the eluate with $Pb(OH)_2$. Samples representing up to 0·5 min. excretion could then be analysed without interference, but the recovery of the pyridone from the adsorption process was not complete, and was not the same in the presence, as in the absence, of urine (see Table 3). Reducing the strength of the NaOH used for elution caused some improvement in the recovery of *N*-methyl-2-pyridone-5-carbonamide but swelling of the Lloyd's reagent occurred when the normality of the NaOH was <0·2, rendering difficult the removal of the eluate. The use of two successive elutions with NaOH did not appreciably improve the recovery. The method finally adopted involved one elution with 0·2 N-NaOH, errors due to incomplete recovery being avoided by the use of internal standards (see p. 517).

Correction for non-specific colour in urine. When the colour reaction was applied directly to urine, a relatively small amount of a reddish colour was produced, in addition to the brownish yellow due to the reagents, the purple due to *N*-methyl-2-pyridone-5-carbonamide and the orange due to *N*-methylnicotinamide. The amount of this reddish colour was much less in Decalso eluates, or in urines treated with Lloyd's reagent and $Pb(OH)_2$, than in untreated urine. That this colour was not derived from acid amides was shown by the fact that it was produced by the diazotization process alone. Since it absorbed at 500 mμ., and also to a slight extent at 590 mμ., its presence would lead to error in the determination of nicotinic acid metabolites in urine unless an appropriate blank determination were made.

Various methods were tested for the production of the red colour in the absence of the colours due to nicotinic acid metabolites. In most cases, the omission of one of the reagents required for the hypobromite reaction, or an alteration in the order of addition of the reagents either led to precipitation of the bromine substitution product of phenol or seriously affected the intensity of the reagent blank. Procedures which were satisfactory in these respects did not completely prevent colour formation by nicotinic acid derivatives. The method finally adopted involved the use of half-strength hypobromite and the addition of the phenol before the hypobromite. Under these conditions only about 0·3% of the *N*-methyl-2-pyridone-5-carbonamide colour, and 13% of the *N*-methylnicotinamide colour, developed.

Table 2. *Colour development by* N*-methyl-2-pyridone-5-carbonamide in the presence of normal human urine*

Pre-treatment of urine	Amount of urine added (in min. excretion)	Colour development (%)	No. of urine specimens tested
—	None	100	—
$Pb(OH)_2$	0·0125	102	1
$Pb(OH)_2$	0·025	100–103	3
No treatment	0·05	92–100	4
$Pb(OH)_2$		99–100	3
No treatment	0·1	85–95	4
$Pb(OH)_2$		97–105	7
$Pb(OH)_2$	0·2	94–101	2

Table 3. *Recovery of* N*-methyl-2-pyridone-5-carbonamide from Lloyd's reagent in the presence and absence of urine*

	Percentage recovered from Lloyd's reagent			
	2 Elutions with 0·05 N-NaOH	2 Elutions with 0·1 N-NaOH	1 Elution with 0·2 N-NaOH	1 Elution with 0·5 N-NaOH
Urine present	89·1	89·1	87·0	68·0
Urine absent	73·8	71·5	70·2	60·0

Methods for determination of N*-methyl-2-pyridone-5-carbonamide in urine*

Rapid method (applicable if rate of excretion exceeds 50 mg./24 hr.). The following reagents are required in addition to those already given; M-$Pb(NO_3)_2$ solution; 0·1 N borax solution.

Twenty-four hr. specimens of urine were collected under toluene and were stored at room temperature until required for analysis. A suitable sample of urine (1 min. excretion) was measured into each of two centrifuge tubes (1 and 2). To tube 2 a known amount of *N*-methyl-2-pyridone-5-carbonamide was added (usually 50 μg.); to both tubes 2 ml. M-$Pb(NO_3)_2$ solution was added, followed by sufficient 2·5 N-NaOH solution to form $Pb(OH)_2$ and to raise the pH value of the solution finally to about 9·2. The pH was tested by external use of indicators or indicator papers, with 0·1 N borax solution as standard. The NaOH solution (about 1·3 ml.) was added slowly with frequent mixing, and the mixture was shaken at intervals for 15 min. thereafter, the pH being checked at intervals. The volume in each case was made to 10 ml. with water and the solution mixed, centrifuged and filtered (Whatman no. 40, 7 cm., paper); 1 ml. of the filtrate from tube 1 was transferred to each of two $6 \times \frac{5}{8}$ in. test tubes (test solution and test solution blank) and 1 ml. filtrate from tube 2 to each of a further two test tubes (recovery and recovery blank). To each test tube were added 3 ml. water. The colour reaction was applied to the test and recovery solutions in the manner described previously, using hypobromite (*a*). In the case of the two blank solutions, the hypobromite was diluted 1:1 with water before use and the phenol added 4 min. before the hypobromite. The extinction (*E*) of each solution was measured in a spectrophotometer against distilled water at 590 mμ., 1 hr. after addition of the coupling agent.

Calculation of results. Excretion of *N*-methyl-2-pyridone-5-carbonamide (mg./24 hr.)

$$= \frac{(T - B_t)}{(R - B_r) - (T - B_t)} \times 1{\cdot}44 \times \frac{A}{M},$$

where T and R are extinctions (E values) of test and recovery, B_t and B_r are extinctions (E values) of test and recovery blanks, A = amount (μg.) of test substance added for recovery, and M = number of minutes excretion to which the sample of urine taken is equivalent. To express the result in terms of nicotinic acid, it was multiplied by the factor 0·809.

General method for N*-methyl-2-pyridone-5-carbonamide in urine.* The following solutions are required in addition to those already listed: 3% (v/v) acetic acid; 0·1 and 2·0 N-HCl; Lloyd's reagent (Eli Lilly and Co.); 0·2 N-NaOH.

A suitable sample of urine (2 min. excretion in the case of normal urines, less if the rate of excretion exceeded 50 mg./24 hr.) was measured into each of two graduated centrifuge tubes (1 and 2) and to tube 2 a known amount of *N*-methyl-2-pyridone-5-carbonamide was added (usually 25 μg.); 0·5 ml. 3% acetic acid was then added to each tube and sufficient water to bring the total volume to 10 ml. 2 N-HCl (0·5 ml.) and 0·2 g. Lloyd's reagent were added to each tube and the tubes were stoppered, shaken for 10 min., and centrifuged. The supernatant fluid was poured off and the tubes were inverted to drain. The Lloyd's reagent in the tubes was washed by adding 5 ml. of 0·1 N-HCl to each tube, mixing the contents, centrifuging and pouring off the supernatant liquid. The tubes were then drained for 10 min. Any fluid remaining at the necks of the tubes was wiped off, and 4 ml. 0·2 N-NaOH were added. The tubes were stoppered, shaken for 10 min., and centrifuged. After it had been checked that the total volume was exactly the same in the two tubes (usually 4·5 ml.), 3 ml. of the supernatant fluid were in each case transferred to another centrifuge tube containing 2 ml. M-$Pb(NO_3)_2$ solution. The pH was brought to 9·2 with 2·5 N-NaOH (about 1·2 ml.), the volume made to 10 ml. with water, and the solution centrifuged and filtered as described in the previous section. Two 3 ml. samples of the filtrate from tube 1 were taken for analysis (test and test blank) and two 3 ml. samples from tube 2 (recovery and recovery blank); 1 ml. water was added to each tube. The procedure for colour development, measurement, and calculation of results was the same as that described in the previous section.

Determination of N*-methylnicotinamide in urine*

Decalso (Permutit Co. Ltd.) was activated by the method of Hochberg *et al.* (1945); 0·067 M phosphate buffer (pH 7) was prepared by mixing 40 ml. KH_2PO_4 solution (9·078 g./l.) and 60 ml. Na_2HPO_4 solution (9·465 g. anhydrous Na_2HPO_4/l.); 25% (w/v) KCl solution.

Twenty-four hr. specimens of urine were collected under toluene, and, if not analysed at once, were preserved with glacial acetic acid (10 ml./24 hr. excretion) and stored in a refrigerator. A suitable sample of urine (4 min. excretion in the case of normal urines, less if the rate of excretion exceeded 20 mg. *N*-methylnicotinamide chloride/24 hr.) was measured into each of two test tubes (1 and 2). To tube 2 a known amount of *N*-methylnicotinamide chloride was added (usually 50 or 100 μg.). To both tubes 15 ml. buffer solution (pH 7) were added and sufficient water to bring the volume to 30 ml. The solution was in each case poured on to an adsorption column (see Hochberg *et al.* 1945) containing 2 g. activated Decalso. The column was washed with three successive 5 ml. lots of water, sucking dry after each washing. The *N*-methylnicotinamide adsorbed on each column was eluted with hot 25% (w/v) KCl solution and exactly 10 ml. of the eluate were collected in each case. Two 2 ml. samples of the eluate from column 1 (test and test blank) and two 2 ml. samples of the eluate from column 2 (recovery and recovery blank), were taken for colour development. Further 2 ml. samples of each eluate were measured out and titrated with 0·2 N-NaOH to the phenolphthalein end point to determine the amount of alkali required for neutralization. The appropriate amount of 0·2 N-NaOH (0·05–0·1 ml.) was added to each sample taken for analysis, before making the total volume to 4 ml. with water. The procedure for colour development, measurement and calculation of results was the same as that described in previous sections, except that hypobromite (*b*) was used and spectrophotometer readings were taken at 500 mμ. after 1 hr. The factor 0·794 was used to convert the result into *N*-methylnicotinamide ion, and the factor 0·713 to express it in terms of nicotinic acid.

Identification of metabolites on filter-paper chromatograms

Filter-paper chromatography has been successfully used for the separation of a number of nicotinic acid metabolites in urine (see Reddi & Kodicek, 1953), but up till the present time no suitable colour reaction has been available for the

detection of *N*-methyl-2-pyridone-5-carbonamide on paper chromatograms. The possibility of using the present reaction for this purpose was therefore investigated. Preliminary experiments showed that azo colours could be produced on filter paper from all the nicotinic acid metabolites capable of giving the reaction in solution. The addition of phenol was not required, the filter paper itself apparently acting as an agent for the removal of excess of bromine. The diazotization and coupling technique could be simplified, without affecting the results, by applying a mixture of HCl and $NaNO_2$, followed by a mixture of ammonium sulphamate and coupling agent. By the use of an 80% (v/v) aqueous solution of propanol as chromatographic solvent, it was possible to separate and identify each of the component substances in mixtures containing *N*-methyl-2-pyridone-5-carbonamide, *N*-methylnicotinamide and nicotinamide. The pyridone could also be readily identified in urine, but attempts to demonstrate the presence of *N*-methylnicotinamide and nicotinamide were unsuccessful.

Samples of urine were concentrated tenfold by evaporation *in vacuo*, and four successive 0·01 ml. portions applied to a sheet of Whatman no. 1 filter paper, the paper being dried thoroughly after each application (see Reddi & Kodicek, 1953). In the case of other solutions a single 0·01 ml. portion was applied. After development of the chromatogram with 80% propanol the paper was left to dry. When all traces of the solvent had disappeared, light was excluded and the paper was sprayed with hypobromite solution (*a*) (see p. 515), left for 15 min., and heated for 5 min. in a hot air oven at about 100°. It was then sprayed, first with a mixture of equal parts of 2·4 N-HCl and 0·1% $NaNO_2$, and 1 min. later with a mixture of equal parts of 0·5% ammonium sulphamate and 0·1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride solution. The mixtures for spraying were prepared immediately before use and the paper sprayed lightly to minimize spreading of the spots.

Under the conditions described above, R_F values of 0·24 for *N*-methylnicotinamide, 0·60 for *N*-methyl-2-pyridone-5-carbonamide, and 0·72 for nicotinamide were observed. As little as 1 μg. of *N*-methyl-2-pyridone-5-carbonamide or 2 μg. of nicotinamide or *N*-methylnicotinamide, could be detected.

RESULTS AND DISCUSSION

Accuracy of the methods

The relationship between the extinction (*E* value) of the colour formed and the amount of the test substance taken for colour development was very nearly linear in all cases. Typical calibration curves for the two pyridones and for *N*-methylnicotinamide and nicotinamide are shown in Fig. 2. The precision of the method for colour development was tested by developing and reading a series of twelve tubes each containing 25 μg. of *N*-methyl-2-pyridone-5-carbonamide, and a similar number containing 25 μg. of *N*-methylnicotinamide. The standard deviation of *E* values from the mean was $\pm 1{\cdot}1$% for the pyridone and $\pm 0{\cdot}5$% for *N*-methylnicotinamide.

Although urea reacts with hypobromite, the presence of amounts up to 4 mg. had no appreciable

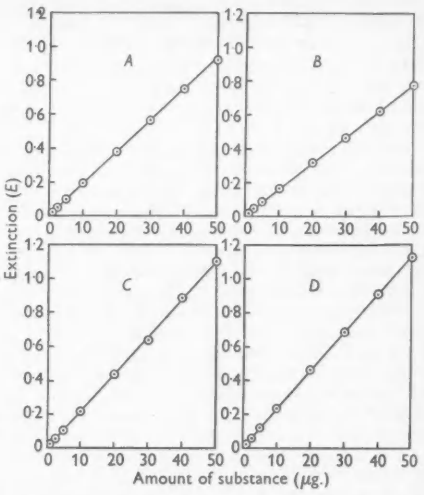

Fig. 2. Relationship between amount of substance taken for analysis and the extinction (*E*) of the colour produced. Readings taken in a Beckman spectrophotometer using 1 cm. cells. *A*, *N*-methyl-2-pyridone-5-carbonamide read at 590 mμ.; *B*, *N*-methylnicotinamide chloride at 500 mμ.; *C*, *N*-methyl-2-pyridone-3-carbonamide at 590 mμ.; *D*, nicotinamide at 500 mμ.

effect on colour formation by *N*-methyl-2-pyridone-5-carbonamide. Alcohols (e.g. ethanol and propanol) interfered with the diazotization process, giving an evanescent red colour. Azo colours were produced by various primary aromatic amines, but could be distinguished from the colours due to acid amides, since hypobromite treatment was not required for their formation.

In applying the methods to urine it was necessary to correct, by means of internal standards, for interference from other urinary constituents or for incomplete recovery from adsorbents. This procedure prevented a systematic error, but reduced the reproducibility of individual results. In the case of the method for *N*-methyl-2-pyridone-5-carbonamide, the variation between duplicates amounted to 1–5%. The method for *N*-methylnicotinamide was less precise, due principally to variability in the recovery from the adsorption process, but also to the relatively high blank. Since the average variation between duplicates was 14%, it was essential for all determinations of this metabolite to be made in duplicate and the mean taken.

Comparison with other methods

The present method for *N*-methyl-2-pyridone-5-carbonamide is superior both in speed and accuracy to the method of Holman & Lous (1951). The

method for *N*-methylnicotinamide has the advantages that it does not require the use of fluorimetric apparatus, and that it can be used in conjunction with the method for the pyridone with little alteration in reagents, technique or equipment. A series of urine samples was analysed by the present methods, and also by the fluorimetric method of Carpenter & Kodicek (1950) for *N*-methylnicotinamide, and by the nitration method of Holman & Lous (1951) for *N*-methyl-2-pyridone-5-carbonamide. The results (Table 4) show satisfactory agreement between methods, but there was a tendency in the case of the pyridone for values to be slightly lower by the azo than by the nitration method.

Application of the methods

The present methods were used to investigate the comparative effects of dosing with nicotinic and quinolinic acids on the excretion of nicotinic acid metabolites by man. Henderson (1949) showed that the administration of large amounts of quinolinic acid to rats caused an increase in the excretions of nicotinic acid and *N*-methylnicotinamide. Krehl, Bonner & Yanofsky (1950) and Reddi & Kodicek (1953) confirmed these results for the rat, but did not extend their observations to man. They concluded that the conversion of quinolinic acid into nicotinic acid and its metabolites by the rat probably represents a side reaction rather than a main pathway in nicotinic acid metabolism.

The urinary excretions of *N*-methyl-2-pyridone-5-carbonamide and *N*-methylnicotinamide by an adult subject (W.I.M.H., aged 38 years) receiving a normal diet were determined before and after oral and intravenous administration of 300 mg. of quinolinic acid, and before and after oral administration of an equivalent amount of nicotinic acid (221 mg.). In the case of two further subjects (A.B.M., aged 30, and E.M.W., aged 42 years) the excretion of each metabolite was determined before and after oral administration of 600 mg. of quinolinic acid. Nicotinamide and nicotinic, and nicotinuric, acids could not be determined by the present methods, but it has been shown that, although they may be excreted after dosing with nicotinic acid (Reddi & Kodicek, 1953), the total amounts excreted do not represent a very large proportion of the dose administered, the combined excretions of acid-hydrolysable derivatives (nico-

Table 4. *Comparison between results obtained by different methods of analysis*

Urine specimen	*N*-Methylnicotinamide ion excretion (mg./24 hr.) found by: Proposed method	*N*-Methylnicotinamide ion excretion (mg./24 hr.) found by: Method of Carpenter & Kodicek (1950)	*N*-Methyl-2-pyridone-5-carbonamide excretion (mg./24 hr.) found by: Proposed method	*N*-Methyl-2-pyridone-5-carbonamide excretion (mg./24 hr.) found by: Method of Holman & Lous (1951)
1	6·0	5·9	13·6	14·6
2	30·9	31·7	101	101
3	—	—	14·4	15·6
4	9·5	10·0	13·8	14·3
5	4·4	4·8	16·9	18·0

Table 5. *Comparative effects of administration of quinolinic and nicotinic acids on the urinary excretions of N-methylnicotinamide and N-methyl-2-pyridone-5-carbonamide by humans*

Subject	Dose administered	Urinary excretion of *N*-methylnicotinamide ion (I) (mg./24 hr.): Before dose Day 1	Before dose Day 2	After dose Day 1	After dose Day 2	Urinary excretion of *N*-methyl-2-pyridone-5-carbonamide (II) (mg./24 hr.): Before dose Day 1	Before dose Day 2	After dose Day 1	After dose Day 2	Total excretion of I and II (mg./24 hr.) expressed as nicotinic acid: Before dose Day 1	Before dose Day 2	After dose Day 1	After dose Day 2
W.I.M.H.	Quinolinic acid (300 mg.), orally	6·0	6·8	9·3	7·5	13·6	15·4	14·4	14·5	16·4	18·6	20·0	18·5
W.I.M.H.	Quinolinic acid (300 mg.), intravenously	—	7·5	8·4	8·9	—	14·5	15·2	19·7	—	18·5	19·9	23·9
W.I.M.H.	Nicotinic acid (221 mg.), orally	—	4·8	30·9	13·0	—	15·3	101	53·1	—	16·8	109	54·7
A.B.M.	Quinolinic acid (600 mg.), orally	9·5	10·4	16·2	15·7	13·8	13·6	13·8	19·2	19·8	20·3	25·7	29·6
E.M.W.	Quinolinic acid (600 mg.), orally	4·4	5·1	4·5	5·6	16·9	13·5	11·3	11·9	17·7	15·5	13·2	14·7

tinic acid, nicotinuric acid and nicotinamide) in the first 72 hr. after ingestion of 500 mg. nicotinic acid being only 16–30% of the dose (Holman & Lange, 1950). The quinolinic acid used was supplied by Messrs L. Light and Co. It was further purified via the dimethyl ester and finally recrystallized twice from hot water. The experimental results are shown in Table 5.

It is clear that the administration, either orally or intravenously, of a comparatively large dose of quinolinic acid had very little effect on the excretions of *N*-methylnicotinamide and pyridone. The results for subjects W.I.M.H. and A.B.M. show some indication of a slight increase in the levels of excretion, but the effect is insignificant in comparison with that observed after administration of nicotinic acid. In the case of subject W.I.M.H., nicotinic acid was 37 times as effective as quinolinic acid in raising the total excretion of *N*-methylnicotinamide and pyridone.

The results support the conclusion that in man, as well as in the rat, quinolinic acid is probably not an important intermediate in nicotinic acid metabolism.

SUMMARY

1. Since the main metabolites of nicotinic acid normally excreted in human urine are acid amides, the possibility was investigated of developing a colorimetric method for their determination in urine, based on conversion of the carbonamide into an amino group by heating with hypobromite, followed by diazotization and coupling of the amino compound formed to yield an azo dye.

2. It was found that when the excess of hypobromite was suitably controlled, and *N*-(1-naphthyl)-ethylenediamine dihydrochloride was used as coupling agent, azo colours could be produced from *N*-methyl-2-pyridone-5-carbonamide, *N*-methyl-2-pyridone-3-carbonamide, *N*-methylnicotinamide and nicotinamide.

3. Optimum conditions for colour production were ascertained and a method worked out for the determination of amounts of from 1 to 50 μg. of each of the colour-producing substances.

4. The removal from urine of substances which interfere with the colour reaction was investigated, and procedures are described which were found suitable for the determination of *N*-methyl-2-pyridone-5-carbonamide and *N*-methylnicotinamide in human urine.

5. Using the present methods, the urinary excreations of nicotinic acid metabolites by three adult subjects were determined before and after administration of quinolinic acid. The results showed evidence of no more than a slight conversion of quinolinic into nicotinic acid.

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Concentration Gradients of Lactate, Hydrogen and some other Ions Across the Intestine *in vitro*

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Previous work (Wilson & Wiseman, 1953*b*) has shown that 50–80% of the glucose which disappears from the mucosal side of sacs of rat intestine *in vitro* is converted into lactic acid and that the lactate appears in a higher concentration on the serosal than on the mucosal side. The experiments to be presented in this paper show that the hydrogen and lactate ions produced by the mucosa migrate in different directions, the hydrogen ions appearing on the mucosal side and the lactate ions mainly on the serosal side. A study has also been made of the associated movements of bicarbonate, chloride, sodium and potassium ions. A preliminary communication of this work has appeared elsewhere (Wilson, 1953).

EXPERIMENTAL

In vitro *preparation of rat intestine (according to the method of Fisher & Parsons).* Oxygenated bicarbonate-saline (Krebs & Henseleit, 1932) containing 0·5% (w/v) glucose was circulated on each side of a segment (60 cm.) of rat small intestine, which had previously been washed with glucose-free bicarbonate-saline, according to the method of Fisher & Parsons (1949*a*). The inner circuit (mucosal side) contained 65 ml. of bicarbonate-saline with a hydrostatic pressure of 30 cm. of water; the outer circuit (serosal side) contained 50 ml. Some glucose-free washing saline remained in the lumen of the intestine at the time it was attached to the circulation unit with a consequent dilution of the glucose in the 65 ml. of added bicarbonate-saline. An initial sample (15 ml.) was removed from both inner and outer solutions after circulation for 2 min., the time required for complete mixing. The initial volume on each side was calculated from the dilution of the glucose in the saline and the volume of fluid added to each side. After 1 hr., 15 ml. samples were removed from both circuits for analysis, the remaining solution was immediately drained and the circuits were washed out with glucose-free saline. The final volume was calculated from the glucose concentration in the 1 hr. sample and the total amount of glucose in the remaining saline plus washings.

The length of the relaxed intestine was measured at the end of each experiment before mincing. To enable calculations on the basis of dry wt., sixteen experiments were performed in which the dry wt. and length were measured. The average dry wt. was 10·0 mg./cm. intestine (range 7·0–13; S.D. 1·7). No difference was found between jejunal and ileal segments.

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Sacs of everted intestine. Samples of rat or golden hamster intestine from 5 to 10 cm. in length were everted, filled with fluid (usually 1·0 ml.) with a blunt needle attached to a syringe and tied at both ends as previously described (Wilson & Wiseman, 1953*a*, *b*). The tissue was kept in ice-cold saline during the preparation of the sacs. Large conical Warburg flasks (125 ml.) were used for the incubation of the sacs in most aerobic and all anaerobic experiments. In a few aerobic experiments, 150 ml. flasks of the type described by Krebs (1933) which were gassed continuously replaced the Warburg flasks. Bicarbonate-saline (Krebs & Henseleit, 1932) containing 0·3% (w/v) glucose was used for the suspending medium (mucosal side) and for the fluid inside the sac (serosal side). The flasks were gassed with 5% CO_2 + 95% O_2 aerobically and 5% CO_2 + 95% N_2 anaerobically. Phosphorus was placed in the centre well of the Warburg flask in anaerobic experiments.

The initial volume on the serosal side was that injected into the sac after careful blotting of the open end. The volume of fluid in the sac after incubation was determined by weighing the sac before and after emptying. The final volume on the mucosal side was calculated from the difference between the initial volume on the mucosal side and the gain in fluid on the serosal side.

Chemical methods. Glucose was measured by the method of Hulme & Narain (1931) on $Cd(OH)_2$ filtrates (Fujita & Iwatake, 1931) in experiments with the Fisher & Parsons apparatus and by the method of Nelson (1944) in all others. Lactic acid was estimated by the method of Barker & Summerson (1941), inorganic phosphate by the method of Berenblum & Chain (1938). Chloride was determined by the Volhard procedure. Bicarbonate was determined manometrically by equilibrating 0·5 ml. bicarbonate solution and 2·0 ml. water with 5% CO_2 + 95% O_2 and adding 0·5 ml. 3N-H_2SO_4 from the side arm. Sodium and potassium were measured with the flame photometer of Domingo & Klyne (1949). The pH was calculated from the measured bicarbonate concentrations, assuming 5% CO_2 (1·14 mM) as gas phase and apparent pK_1 of carbonic acid as 6·10. It was assumed that this concentration of CO_2 was in equilibrium with the bicarbonate solution on each side of the intestinal wall.

RESULTS

Rat intestine (method of Fisher & Parsons)

Lactate and bicarbonate gradients. As shown in Table 1, about one-half of the glucose which disappeared from the mucosal side in five experiments appeared on the serosal side and about one-quarter was converted into lactic acid. The total amount of lactate found on the serosal side was 4–10 times that on the mucosal side. Table 2 shows the con-

Table 1. *Glucose transport and lactate production in rat intestine (method of Fisher & Parsons)*

(Bicarbonate-saline containing about 0·5% glucose was circulated on the two sides of the intestine for 1 hr. at 38°. Gas phase 5% CO_2 + 95% O_2.)

Expt. no.	Glucose lost from mucosal side (μmoles)	Glucose transported to serosal side (μmoles)	Glucose converted into lactate (μmoles)	Lactate on serosal side (μmoles)	Lactate on mucosal side (μmoles)
1	580	270	176	273	79
2	811	547	180	322	38
3	1065	440	256	468	44
4	1088	475	259	450	68
5	600	339	234	391	77

Table 2. *Concentrations of bicarbonate and lactate developed across rat intestine (method of Fisher & Parsons)*

(Bicarbonate-saline containing about 0·5% glucose was circulated on the two sides of the intestine for 1 hr. at 38°. Gas phase 5% CO_2 + 95% O_2. pH calculated taking pK of carbonic acid = 6·10 and CO_2 = 1·14 mM. Two other experiments gave similar results.)

	Lactate concn. (mM)			Bicarbonate concn. (mM)			pH (calculated)		
Expt. no.	1	2	3	1	2	3	1	2	3
Mucosal side (initial)	0	0	0	23·7	22·5	27·3	7·42	7·39	7·48
Mucosal side (final)	1·53	1·07	1·10	16·6	13·2	12·0	7·26	7·16	7·02
Serosal side (initial)	0	0	0	24·0	24·3	30·4	7·42	7·43	7·53
Serosal side (final)	6·60	6·33	10·2	23·0	25·0	30·0	7·46	7·44	7·52

Table 3. *Comparison between lactate produced and bicarbonate decomposed in rat intestine (method of Fisher & Parsons)*

(Bicarbonate-saline containing about 0·5% glucose circulated on the two sides of the intestine for 1 hr. Lactate and bicarbonate estimated in the solutions on each side of the intestine, lactate only estimated in the gut wall.)

		Lactate produced			Bicarbonate lost	Lactate produced/ bicarbonate lost
Gut no.	Length of intestine (cm.)	Mucosal and serosal sides (μmoles)	Gut wall (μmoles)	Total (μmoles)	Mucosal and serosal sides (μmoles)	(%)
1	68	352	77	429	479	90
2	62	360	45	405	356	113
3	85	512	99	611	966	63
4	67	518	98	616	922	67
5	61	468	105	573	550	104
Mean	—	—	—	—	—	87

Table 4. *Movement of substances across the perfused rat intestine (method of Fisher & Parsons)*

(Bicarbonate-saline containing glucose circulated on the two sides of rat intestine (62 cm. in length; 620 mg. dry wt.) for 1 hr. at 38°. See Tables 1 and 2 (Expt. 1) for bicarbonate and lactate values in this experiment.)

		Glucose		Sodium		Potassium		Phosphate		Chloride	
	Volume (ml.)	Concn. (mM)	Total amount (μmoles)	Concn. (mM)	Total amount (μmoles)	Concn. (mM)	Total amount (μmoles)	Concn. (mM)	Total amount (μmoles)	Concn. (mM)	Total amount (μmoles)
Mucosal side (initial)	60·5	24·2	1460	148	8940	6·14	371	1·18	71·5	130	7860
Mucosal side (final)	51·7	17·0	880	146	7550	6·75	349	1·39	72	136	7040
Serosal side (initial)	35·8	27·7	990	141	5040	6·09	218	1·19	42·6	128	4580
Serosal side (final)	41·4	30·4	1260	140	5810	5·16	214	1·40	58·1	118	4890
Change on mucosal side	−8·8	—	−580	—	−1390	—	−22	—	+1	—	−820
Change on serosal side	+5·6	—	+270	—	+770	—	−4	—	+15	—	+310

centration gradients of lactate and bicarbonate developed across the intestinal wall. The bicarbonate concentration on the serosal side did not change appreciably, in spite of the presence of the large amount of lactate; the concentration on the mucosal side fell to about half its initial value, although only a small amount of lactate was present on this side. A striking feature of these experiments is that while most of the lactate ions appeared on the serosal side virtually all of the acidification occurred on the opposite side.

A comparison was made between the lactate produced and the bicarbonate decomposed to determine whether any other acid was formed.

To estimate the lactate in the gut wall, the entire intestine after 1 hr. in the circulation unit was finely minced in a tissue blender with an ice-cold solution of $CdSO_4$. Lactate was estimated in the protein-free filtrate after copper/lime treatment. Filtrates prepared from intestines removed from two rats and immediately minced in the cold served as controls.

The average concentration found in the intestine after 1 hr. in the circulation units was 30 mM. The intestine removed directly from the animal contained 6·9 mM. No information is available on the bicarbonate content of the gut wall owing to the experimental difficulty of estimating bicarbonate in the tissue during incubation. Table 3 shows that an average of 87% (from 63 to 113%) of the acid production (bicarbonate displaced) may be accounted for as lactate.

Shapiro (1952), on the basis of experiments with a somewhat different *in vitro* method, showed that large amounts of some substance other than lactate is formed by the rat intestine but this was not found in the present work. Of the glucose which disappeared (Table 1, Expt. 1) from the mucosal side (580 μmoles), about half was transported across the wall (270 μmoles) and one-quarter converted into lactic acid (176 μmoles). Fisher & Parsons (1953) have shown that 0·165 mg. glucose/cm. of intestine accumulates in the wall under conditions used in these experiments. The 68 cm. of intestine used in this experiment would have accumulated 62 μmoles of glucose, assuming the value of Fisher & Parsons given above. Assuming a Q_{O_2} of -20 (Wilson & Wiseman, 1953*c*) and a R.Q. of 1·0 (and 10 mg. dry wt./cm. of intestine), 101 μmoles of glucose would be completely oxidized. The total glucose accounted for in this way (609 μmoles) agrees fairly well with the measured glucose disappearance (580 μmoles). Similar results were obtained from other experiments.

Movement of other substances across the intestine. Results on the movement of water, glucose, sodium, potassium, phosphate and chloride are shown in Table 4. It might be thought that the movement of fluid across the intestinal wall, in this experiment,

Table 5. *Movement of substances across the wall of a sac of everted rat intestine*

(Sac of everted ileum incubated for 105 min. with bicarbonate-saline in 150 ml. flat-bottomed flask, gassed continuously with 5% CO_2+95% O_2 saturated with water. 276 mg. wet wt., 50·7 mg. dry wt.)

	Volume (ml.)	Glucose		Bicarbonate		Lactate		Chloride		Sodium		Potassium	
		Concen. (mM)	Total amount (μmoles)	Concen. (mM)	Total amount (μmoles)	Concen. (mM)	Total amount (μmoles)	Concen. (mM)	Total amount (μmoles)	Concen. (mM)	Total amount (μmoles)	Concen. (mM)	Total amount (μmoles)
Mucosal side (initial)	25·00	11·7	298	24·3	620	0	0	123	3140	154	3930	5·72	146
Mucosal side (final)	23·33*	4·92	117	14·7	350	6·40	149	127	3020	154	3660	6·66	158
Serosal side (initial)	1·00	11·7	11·7	24·3	24·3	0	0	123	123	154	154	5·72	5·7
Serosal side (final)	2·67	16·9	45·1	24·4	65·0	56·0	150	80	214	147	392	5·58	14·9
Change on mucosal side	$-1·67$*	—	-181	—	-270	—	$+149$	—	-120	—	-270	—	$+12$
Change on serosal side	$+1·67$	—	$+33·4$	—	$+40·7$	—	$+150$	—	$+91$	—	$+238$	—	$+9·2$

* Calculated values. See text.

as shown by the increase in volume on the serosal side (5·6 ml.), was due to the pressure of 30 cm. of water on the mucosal side, but since similar movements of water occur in sacs of everted intestine (see Table 5) where the pressure relationships are reversed it may be taken that the hydrostatic pressure in this preparation is not a major cause of the water movement. The loss of water of 3·2 ml. is partly due to swelling of the tissue (Fisher & Parsons, 1953) and partly due to systematic errors in the calculations of the volumes.

The concentration gradient of glucose developed across the wall and the quantity of glucose transported are similar to those reported by Fisher & Parsons (1949*a*, *b*).

There was a net movement of sodium across the wall of the intestine without any appreciable change in concentration on the two sides. There was no transport of potassium; the concentration on the mucosal side increased owing to the loss of water and decreased on the serosal side owing to the gain in fluid. There was a small net loss of potassium from both solutions, presumably due to an uptake of this ion into the tissue. In the two other experiments in which potassium was estimated, a similar small loss from the solutions was noted. There was a gain in phosphate on both sides indicating loss from the tissue. The decrease in chloride concentration on the serosal side and the corresponding increase on the mucosal side is presumably partly connected with the unequal distribution of lactate.

Sacs of rat intestine

Lactate and bicarbonate gradients. The development of concentration gradients in sacs of everted intestine has already been described (Wilson & Wiseman, 1953*b*). In the experiment recorded in Table 5 the final concentration gradient of lactate was 9. Since the volume of fluid on the mucosal side was 9 times that on the serosal side, the total amounts of lactate on the two sides were equal. The final concentration of lactate on the serosal side was 56 mM, comprising more than one-third of the total anions. In spite of this large amount of lactate, the concentration of bicarbonate remained unchanged. If lactic acid instead of lactate ions had been liberated on the serosal side, all of the bicarbonate would have been decomposed and the pH would have fallen to below 6·0. Instead, the pH remained about 7·4. The bicarbonate concentration on the mucosal side fell to about half its initial value. The total lactate produced on the two sides was 299 μmoles, while the total amount of bicarbonate displaced was 229 μmoles. The discrepancy between these values may be due to buffering of the acid by the tissue.

The $Q_{\text{glucose transported}}$ in the same experiment was 3·0 (μl./mg. dry wt./hr.; 22·4 μl. = 1 μmole) and the $Q_{\text{glucose metabolized}}$ was 13. In the five experiments with the method of Fisher & Parsons (Table 1) the average $Q_{\text{glucose transported}}$ was 9 and the average $Q_{\text{glucose metabolized}}$ was 10. The $Q_{\text{glucose transported}}$ values for sacs of rat intestine vary from 2 to 7 (see Wilson & Wiseman, 1953*b*) but were always lower than those obtained with the circulation units of Fisher & Parsons.

Sodium and chloride ions moved across the intestine in a similar manner to that found with the Fisher & Parsons technique. No significant difference in concentration of sodium or potassium was noted on the two sides. There was, again, a large fall in chloride concentration on the serosal side and a rise on the mucosal side, which may be secondary to the unequal distribution of lactate. Potassium was lost from the tissue into both inner and outer solutions in contrast with the results presented with the other preparation of intestine. Three similar experiments (one sac from the jejunum and two from the upper ileum) gave essentially similar results. In two of these, inorganic phosphate was also estimated. There was a loss of phosphate from the tissue of 0·064 and 0·035 μmole/mg. dry wt./hr. No difference in phosphate concentration was found on the two sides. This agrees with the findings of Smyth & Whaler (1953).

Two experiments were performed to test the effect of glucose on the movement of fluid (Table 6). In the absence of glucose there was practically no movement of fluid from mucosal to serosal sides. This is similar to the results obtained under anaerobic conditions in the presence of glucose (Wilson & Wiseman, 1953*b*).

Table 6. *The effect of glucose on the movement of water across sacs of everted intestine*

(The sacs were placed in 25 ml. Warburg flasks in Expt. 1 and in 125 ml. flasks in Expt. 2. Incubation for 90 min. at 37°.)

Expt. no.	Animal	Initial glucose concn. (mg./100 ml.)	Tissue	Dry wt. (mg.)	$Q_{\text{lactic acid}}$ (μl./mg. dry wt./hr.)	Serosal side: Initial volume (ml.)	Serosal side: Final volume (ml.)	Water transported (μl. fluid/mg. dry wt./hr.)
1	Hamster	300	Jejunum	29	—	0·370	0·875	11·6
		None	Ileum	32	—	0·520	0·576	1·2
2	Rat	210	Ileum	140	27	1·00	2·67	8·0
		None	Jejunum	122	3	1·00	1·03	0·2

Table 7. *The effect of anoxia on concentration gradients of lactate and hydrogen ions across sacs of everted rat intestine*

(Sacs of rat ileum were incubated in bicarbonate-saline containing 0·2% glucose for 90 min. at 37°. The initial bicarbonate concentration was the same on both sides of intestine.)

Gas phase	Initial bicarbonate concentration (mM)	Serosal side Final concn. (mM)			Mucosal side Final concn. (mM)			Total lactate produced (μmoles)	Total bicarbonate lost (μmoles)
		Bicarbonate	Lactate	Bicarbonate + lactate	Bicarbonate	Lactate	Bicarbonate + lactate		
5% CO_2 + 95% O_2	25·2	32·0	18·0	50·0	18·7	0·9	19·6	68	111
5% CO_2 + 95% O_2	20·8	28·0	45·2	73·2	8·6	7·1	15·7	252	204
5% CO_2 + 95% N_2	25·2	15·8	7·9	23·7	21·6	1·8	23·4	48	89
5% CO_2 + 95% N_2	24·3	1·9	22·2	24·1	17·8	1·5	19·3	77	175

Anaerobic experiments. Anaerobically the concentration of bicarbonate fell on the serosal side and this fall was roughly equivalent to the rise in lactate concentration, the sum of the two concentrations being equivalent to the initial bicarbonate concentration (Table 7). A lactate gradient appeared anaerobically, similar to that found aerobically.

Sacs of golden hamster intestine

The hamster produces considerably less lactic acid under aerobic conditions than does the rat (Wilson & Wiseman, 1953*c*), but the concentration gradients of lactate across the wall were similar to those of rat intestine (Table 8). In both sacs the concentrations of bicarbonate fell to about half the initial values on the serosal sides while they increased on the mucosal sides. In this respect the hamster differs from the rat intestine. There is a point of special interest in the case of the ileal sac. Bicarbonate (29 μmoles) was transported from the serosal side to the mucosal side against a concentration gradient of more than 2. Three further experiments showed a similar transport of bicarbonate.

Table 9 shows a typical experiment in which the transport of added lactate from mucosal to serosal sides was tested. There is no evidence for the transport of lactate against a concentration gradient; 4 μmoles of lactate were formed by the tissue and appear in both solutions. In the same sac, glucose was transported against a concentration gradient.

DISCUSSION

The lactate and hydrogen-ion gradient across the intestine. The experiments with rat intestine show that glucose is metabolized in the intestinal wall to lactic acid, the lactate ion appearing mainly on the serosal side and the hydrogen ion appearing on the mucosal side. The gradient for hydrogen ions is completely abolished by anaerobic conditions, though the lactate gradient persists under the conditions of the sac experiments. Concentration gradients of lactate have been previously demonstrated across the wall of tied sacs of frog gastric mucosa (Davies, 1948; Davenport, 1952).

A concentration gradient of a weak acid may be explicable by simple physical forces if the membrane is much more permeable to the undissociated acid than to the ionized form and if there is a pH difference across the membrane (see Höber, 1945). This hypothesis, however, cannot explain the lactate gradient across the rat intestine since the pH difference (0·3 unit) is too small to give rise to the observed lactate gradients of 4 to 20. Furthermore, the pH difference observed with sacs of hamster intestine are opposite to those which would favour a gradient of lactate by this kind of mechanism.

Table 8. *Lactate and bicarbonate gradients developed across sacs of everted hamster intestine*

(Intestine incubated with bicarbonate-saline containing 0·2% glucose in 125 ml. Warburg flasks for 100 min. at 37°. Initial volume on the serosal side = 2·0 ml.; initial volume on the mucosal side = 10·0 ml.)

	Jejunum		Ileum	
	Serosal side	Mucosal side	Serosal side	Mucosal side
Initia bicarbonate concn. (mM)	23·9	23·9	23·9	23·9
Final bicarbonate concn. (mM)	11·6	25·0	10·8	25·9
Final lactate concn. (mM)	5·32	0·91	1·38	0·36
Change in bicarbonate (μmoles)	−21	+1	−29	+29
Change in lactate (μmoles)	+12·7	+8·8	+2·1	+3·7
Total lactate formed (μmoles)	21·5		5·8	
Total bicarbonate lost (μmoles)	20·0		0·0	

Table 9. *Lactate gradient developed across a sac of everted hamster intestine after addition of lactate*

(Hamster ileum (22 mg. dry wt.) incubated in bicarbonate-saline in Warburg flask for 90 min. 37°. Gassed with 5% CO_2 + 95% O_2. The initial concentrations of DL-lithium lactate (6·9 mM) and glucose (5·1 mM) were the same on both sides of the intestine.)

	Serosal side	Mucosal side
Lactate		
Final concn. (mM)	10·1	7·9
Initial (μmoles)	3·1	20·8
Final (μmoles)	5·3	22·9
Change (μmoles)	+2·2	+2·1
Glucose		
Final concn. (mM)	14·8	1·83
Initial (μmoles)	2·3	15·1
Final (μmoles)	7·7	5·4
Change (μmoles)	+5·4	−9·7

A possible explanation for the gradient of lactate is the assumption that it is produced by the muscular layer and diffuses more rapidly into the solution on the serosal side of the small intestine. The following data show that this hypothesis is not tenable. The aerobic glycolysis of the smooth muscle of the cat small intestine is of the order of 2 μl./mg. dry wt./hr. (T. H. Wilson, unpublished observations), which is similar to the values obtained in smooth muscle of other organs (Rosenthal & Lasnitzki, 1928). This is only 10–20% of the lactic acid production of the intestinal wall in the present experiments, the major site of the aerobic lactic acid production being the mucosa (Dickens & Weil-Malherbe, 1941).

A question arises whether the lactic acid produced by the mucosa diffuses primarily into the solution on the mucosal side and is secondarily transferred to the other side. Added lactate, however, was not found to be transported against a concentration gradient by the hamster intestine (Table 9). Cori & Cori (1928) found that lactate absorption in the rat *in vivo* was much slower than that of glucose and was proportional to the amount fed.

The mechanism by which concentration gradients of lactate and hydrogen ions are developed across the wall of the intestine remains obscure.

The mechanism of glucose absorption. The preferential discharge of lactate in one direction suggests that the formation of lactic acid may play a role in the absorption of glucose. Hestrin-Lerner & Shapiro (1953) have recently reported experiments with rat intestine in which radioactive glucose placed on the mucosal side is converted into an unidentified derivative, most of which appeared on the serosal side. The experiments reported here suggest that most of this derivative is lactic acid. Fisher & Parsons (1953) have shown that three times as much glucose is utilized by the rat intestine with glucose only on the mucosal side than with glucose on the serosal side. It is possible that the increased utilization represents an increased production of lactic acid. If this inference is correct then lactic acid production would increase during absorption of glucose from the lumen of the intestine. It is suggested that this conversion of glucose into lactate may play an important role in the absorption of glucose, especially in the cat and rat in which high rates of aerobic glycolysis have been demonstrated.

It must be emphasized that *in vitro* preparations of both rat (Fisher & Parsons, 1949*a*, *b*) and hamster intestine (Wilson & Wiseman, 1953*b*) are also capable of transporting glucose as such across the wall against concentration gradients, the hamster intestine transporting 2–5 times as much as that of the rat. A third mechanism for absorption of glucose is passive diffusion in the direction of the concentration gradient. This may be quantitatively unimportant, at least in the guinea pig intestine *in vitro*, since Darlington & Quastel (1953) have shown that with 0·5% glucose on the mucosal side and none on the serosal side the movement of glucose across the wall was inhibited up to 90% by metabolic inhibitors and anaerobic conditions. The relative importance of these three mechanisms (transport of

glucose as such, conversion of glucose into lactate, and passive diffusion of glucose) probably varies considerably with different conditions *in vitro* and *in vivo* and also with different species of animals.

The movement of water and salts across the intestine. The movement of water and salts across the wall of the intestine was regularly observed in the preparations used; in the experiments with everted sacs (Table 5) this movement of fluid was against a slight hydrostatic pressure. There was, however, no evidence of the transfer of sodium or potassium against concentration gradients and no significant difference in the concentration of total ions on the two sides of the intestine was observed (in Table 5 the total concentration of measured ions was 309 m-equiv./l. on the mucosal side and 313 m-equiv./l. on the serosal side). Since the concentration of glucose was 17 mM on the serosal side and 5 mM on the mucosal side, there was probably a difference of osmotic pressure on the two sides. The movement of water and salts was from the side of lower to that of higher osmotic pressure (neglecting the small amounts of protein and undetermined ions on the two sides). In the absence of glucose there was no movement of fluid (Table 6) but this result is not decisive since the tissue may need added glucose as substrate to maintain its normal metabolism. Although there is evidence (Ingraham & Visscher, 1936; Visscher *et al.* 1944) that active processes are involved in the absorption of water and salts *in vivo*, the present *in vitro* experiments may be explained by physical forces.

Secretion of bicarbonate. The movement of bicarbonate across the wall of hamster ileum against a concentration gradient is comparable with that observed with everted frog gastric mucosa secreting hydrochloric acid into bicarbonate solutions (Davies, 1948). In the case of gastric mucosa, bicarbonate also moves across the wall against a concentration gradient, though in the opposite direction from that with hamster ileum.

SUMMARY

1. When glucose-containing saline was circulated through rat intestine according to the method of Fisher & Parsons (1953) the concentration of lactate rose on the serosal side up to 9 times that on the mucosal side within 1 hr. The bicarbonate concentration decreased to about half the original value on the mucosal side while that on the serosal side remained practically constant. Thus glucose is converted into lactic acid, the lactate appearing mainly on the serosal side, the hydrogen ion appearing on the mucosal side.

2. This was confirmed in experiments with sacs of everted intestine of the rat. Concentration gradients of lactate of 6 to 20 were observed. The concentration of bicarbonate decreased on the mucosal side while that on the serosal side remained constant or increased.

3. Anaerobically the bicarbonate concentrations on both sides of the sacs of rat intestine decreased, the decrease being equivalent to the increase in lactate concentration on the two sides. The lactate gradient did not disappear under anaerobic conditions.

4. Possible mechanisms for the lactate gradients are discussed.

5. It is suggested that the conversion of glucose into lactate may play a role in the absorption of glucose *in vivo*.

6. Sacs of everted ileum from the hamster can transfer bicarbonate from serosal to mucosal sides against a concentration gradient.

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